University of Alberta

The role of proteasomal proteolysis in the adrenergic induction of arylalkylamine-N-acetyltransferase in rat pinealocytes

by

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Abstract

In this study, I examined the consequence of inhibiting the proteasome with MG132 and clasto-lactacystin-β-lactone (c-lact), on the adrenergic induction of arylalkylamine-N-acetyltransferase (*aa-nat)* mRNA, protein and enzyme activity in cultured rat pinealocytes using RT-PCR, Western blot and radioenzymatic assay. Addition of MG132 or c-lact one hour prior to or concurrent with norepinephrine (NE) significantly reduced the levels of adrenergic-stimulated *aa-nat* mRNA and protein. Proteasome inhibitors also inhibited *aa-nat* induction when cells were stimulated by dibutyryl cAMP, a cell permeable cAMP analog, thus excluding an effect of proteasome inhibition at the receptor level. The effects of proteasome inhibitors on adrenergic stimulation of *aa-nat* can be abolished by cycloheximide, a protein synthesis inhibitor, indicating that continuous synthesis of a protein repressor may be required for *aa-nat* inhibition. Taken together, these results suggest that a protein repressor, which is regulated by the proteasome, is responsible for suppressing NE induced transcription of *aa-nat*.

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List of abbreviations

5-HT, 5-hydroxytryptamine

AA-NAT, arylalkylamine-N-acetyltransferase

AMC, 7-amino-4-methylcoumarin

AP-1, activator protein-1

CBP, CREB binding protein

C/EBP, CCAAT enhancer binding protein

CHIP, chromatin immunoprecipitation

c-lact., clasto-lactacystin β-lactone

CRE, cAMP response element

CREB, cAMP response element binding protein

CREM, cAMP response element modulator

cyclo, cycloheximide

DAG, diacylglycerol

DbcA, dibutyryl cAMP

DMEM, Dulbecco's modified Eagle's medium

DREAM, downstream regulatory element antagonist modulator

DTT, dithiothreotol

EBSS, Earle's balanced salt solution

FCS, fetal calf serum

Fra-2, Fos-related antigen 2

GABA, γ-amino butyric acid

GAM, goat anti-mouse antibody

GAPDH, glyceraldehyde-3-phosphate-dehydrogenase

GAR, goat anti-rabbit antibody

h, hour(s)

HIOMT, hydroxyindole-O-methyltransferase

HRP, horseradish peroxidase

ICER, inducible cAMP early repressor

IML, intermediolateral cell column

IP_3, inositol triphosphate

KID, kinase inducible domain

MG132, carbobenzoxy-L-leucyl-L-leucyl-L-leucinal

MKP-1, mitogen-activated protein kinase phosphatase-1

MT, melatonin

NAS, N-acetylserotonin

NE, norepinephrine

PBS, phosphate buffered saline

PKA, cAMP dependent protein kinase

PKC, protein kinase C

PLC, phospholipase C

PMA, 4-beta-phorbol-12-myristate-13-acetate

pRB, retinoblastoma tumour suppressor protein

PSF, penicillin-streptomycin-amphotericin B

PVN, paraventricular nucleus

RHT, retinohypothalamic tract

SCG, superior cervical ganglia

SCN, suprachiasmatic nucleus

Suc-LLVY-AMC, N-succinyl-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin

TTBS, Tris-Cl buffered saline with Tween-20

1. Introduction

1.1 Neural pathways that regulate pineal function

The daily rhythm of melatonin production in the pineal gland is a tightly controlled process that is regulated by changes in lighting conditions. A neuronal network involving the retina, the suprachiasmatic nucleus of the hypothalamus (SCN) and the sympathetic neurons of the superior cervical ganglia (SCG) innervates the pineal gland and regulates the release of norepinephrine (NE) at night (Stephan & Zucker, 1972a; Stephan & Zucker, 1972b) (Fig. 1.1). The nightly release of NE stimulates the synthesis of arylalkylamine-N-acetyltransferase (AA-NAT), the rate-limiting enzyme involved in the conversion of serotonin to melatonin (MT). In response to light at the end of the night, NE release by the SCG is inhibited, AA-NAT is degraded, and melatonin production ceases. The changes in environmental lighting levels result in the circadian rhythm of melatonin production, a highly conserved process in vertebrate physiology.

Studies have demonstrated that individual neurons of the SCN contain genetically driven clocks (Welsh, Logothetis, Meister, & Reppert, 1995). A critical feature of the SCN is its ability to be reset by environmental lighting stimuli. Light received by the retina is converted to a neuronal stimulus, which travels to the SCN from the retina via the retinohypothalamic tract (RHT) (Fig. 1.1). The SCN's 24-hour clock is synchronized to the external light-dark cycle by input from the RHT. This synchronization manifests as changes in gene expression and hormone output throughout the body according to the light-dark cycle. The nightly release of melatonin from the pineal gland results from entrainment of the SCN by environmental lighting conditions (Arendt, Deacon, English,

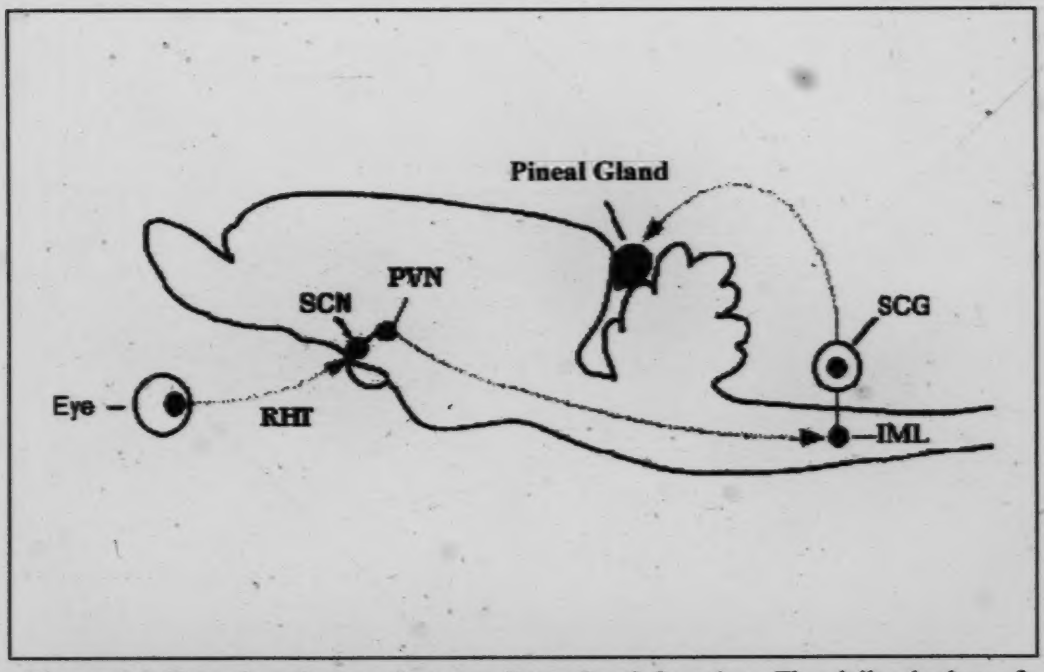

Figure 1.1 Neural pathways that regulate pineal function. The daily rhythm of melatonin production in the pineal gland is regulated by changes in environmental lighting conditions. Light received by the retina is converted to a neuronal stimulus, which travels to the suprachiasmatic nucleus (SCN) from the retina via the retinohypothalamic tract (RHT). The SCN is connected to the pineal gland via a multisynaptic pathway involving neurons of the paraventricular nucleus (PVN), sympathetic neurons of the intermediolateral cell column (IML), and the norephinephrine releasing neurons of the superior cervical ganglion (SCG). Figure modified from Foulkes, Borjigin, Snyder, & Sassone-Corsi, 1997.

Hampton, & Morgan, 1995; Wehr, 1991). For example, the SCN can stimulate the pineal gland to produce melatonin for a longer period when animals are exposed to 10 days of long nights as compared to animals exposed to 10 days of short nights.

The SCN is connected to the pineal gland via a multi-synaptic pathway involving neurons of the paraventricular nucleus (PVN), sympathetic neurons of the intermediolateral cell column, and the NE-releasing neurons of the SCG (Klein et al., 1983; Moore & Klein, 1974) (Fig. 1.1). Current models suggest that during the day, the SCN releases both stimulatory (glutamate) and inhibitory (γ-amino butyric acid, GABA) signals to the PVN at the same time. As a result of the inhibitory signals, the PVN does not stimulate neurons of the SCG, which prevents the pineal gland from being stimulated. Studies support this hypothesis since blocking GABA input to the PVN for 4 hours results in the stimulation of SCG neurons, the release of NE and the subsequent production of melatonin during the day (Perreau-Lenz et al., 2003; Perreau-Lenz, Kalsbeek, Pevet, & Buijs, 2004). Under normal physiological conditions, melatonin is never produced during the day. Data also clearly demonstrates that the release of glutamate from the SCN and PVN is responsible for the stimulation of melatonin production in the pineal gland (Perreau-Lenz et al., 2004).

In response to light, GABA is released from the SCN along with glutamate. The inhibitory effects of GABA mask the stimulatory effects of glutamate, which prevents the activation of SCG neurons. However, during the night or in the absence of light, GABA is not released and glutamate is able to stimulate the PVN → SCG → pineal gland pathway. Ultimately, this complex interaction of neurons and signals culminates in the daily rhythm of melatonin production.

1.2 Biosynthesis of melatonin in the pineal gland

The nightly release of melatonin in the pineal gland is regulated by the enzyme AA-NAT (Klein & Berg, 1970; Klein, 1985). AA-NAT is the rate-limiting enzyme in the conversion of 5-hydroxytryptamine (5-HT or serotonin) to melatonin (N-acetyl-5-methoxytryptamine) (Fig. 1.2). Increased AA-NAT protein and activity levels, in response to NE stimulation at night, results in the acetylation of 5-HT and a large increase in the abundance of N-acetylserotonin (NAS), a precursor to melatonin (Ganguly, Coon, & Klein, 2002; Klein & Weller, 1970). This product is then rapidly converted to melatonin via the enzyme, hydroxyindole-O-methyltransferase (HIOMT), which adds a methyl group to NAS (Fig. 1.2). Levels of HIOMT protein display little to no variation throughout the day/night cycle, suggesting that it is constantly active (Klein & Lines, 1969). Thus, the production of melatonin is completely regulated by the activity of AA-NAT and the availability of NAS, the HIOMT substrate.

1.3 Transmembrane signaling in the pineal gland

The nightly release of NE by the superior cervical ganglion stimulates both the α_1- and β_1-adrenergic receptors in the rat pineal gland (Reiter, 1993) (Fig. 1.3). The binding of NE to β_1-adrenergic receptors causes GTP to bind to the Gα subunit of a G-protein. Once GTP is bound, the Gα subunit dissociates from the G-protein and β_1-receptors (Klein, 1985) (Fig. 1.3). Dissociation of the Gα subunit allows it to interact with adenylyl cyclase, a membrane bound protein that converts ATP to cAMP. As a

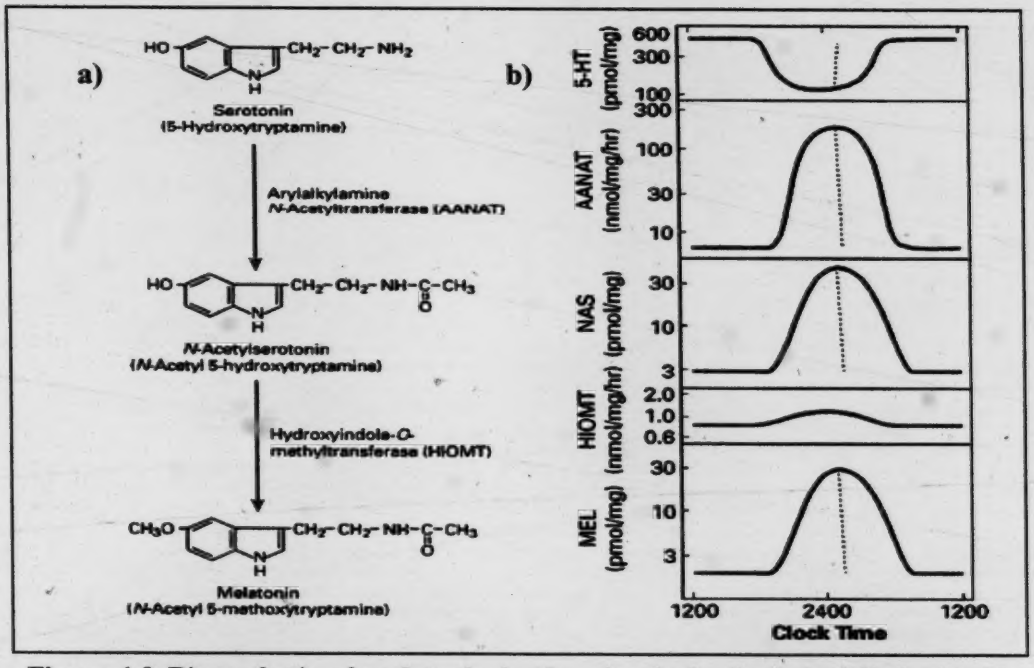

Figure 1.2 Biosynthesis of melatonin in the pineal gland. a) Arylalkylamine-N-acetyltransferase (AA-NAT) is the rate-limiting enzyme in the conversion of 5-hydroxytryptamine (5-HT) to melatonin (N-acetyl-5-methoxytryptamine). Increased AA-NAT protein and activity levels, in response to NE stimulation at night, results in a large increase in the abundance of N-acetylserotonin (NAS). This product is then rapidly converted to melatonin via the enzyme, hydroxyindole-O-methyltransferase (HIOMT), which adds a methyl group to NAS. b) There is a significant day/night rhythm in AA-NAT activity, NAS levels, and melatonin production. However, HIOMT displays very little daily rhythm. Figure modified from Ganguly, Coon, & Klein, 2002.

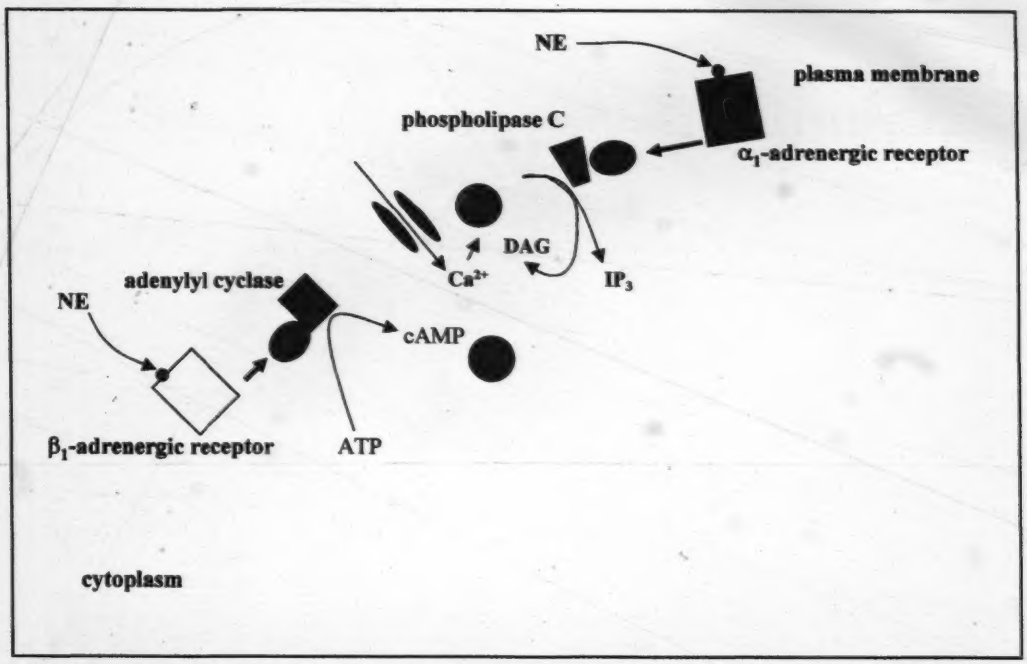

Figure 1.3 Transmembrane signalling in the pineal gland. NE binds to β_1-adrenergic receptors and activates adenylyl cyclase through the action of a G-protein. Activated adenylyl cyclase converts ATP to cAMP resulting in a rise in intracellular cAMP levels and the activation of cAMP dependent protein kinase (PKA). NE also binds to α_1-adrenergic receptors which leads to the activation of phospholipase C and the opening calcium channels. As a result, intracellular calcium levels rise and inositol triphosphate (IP_3) and diacylglycerol (DAG) are produced resulting in the activation of protein kinase C (PKC).

result of NE binding to the β_1-adrenergic receptor, intracellular cAMP levels rise approximately 100-fold through the activation of adenylyl cyclase (Klein, 1985).

α_1-adrenergic receptors also play a role in regulating intracellular cAMP levels (Klein, Sugden, & Weller, 1983; D. Sugden & Klein, 1988) (Fig. 1.3; Fig. 1.4). Stimulation of α_1-receptors alone does not activate adenylyl cyclase, but it does help to potentiate the rise in cAMP caused by NE binding to β_1-adrenergic receptors. It has been shown that potentiation of the cAMP response by α_1-adrenergic receptors works through the actions of protein kinase C (PKC) (Ho, Chik, & Klein, 1988a; Ho, Chik, & Klein, 1988b; Ho, Thomas, Chik, Anderson, & Klein, 1988; D. Sugden & Klein, 1988; L. A. Sugden, Sugden, & Klein, 1987). Experimental evidence determined that the potent activator of PKC, 4-β-phorbol-12-myristate-13-acetate (PMA), causes a further increase in cAMP levels, but only after β_1-adrenergic receptor activation.

NE activates PKC by causing a rise in intracellular Ca^{2+} and by activating phospholipase C (PLC), a membrane bound enzyme that cleaves phosphatidyl inositol to yield diacylglycerol and inositol triphosphate (Ho & Thomas et al., 1988; Ho, Chik, & Klein et al., 1988b; L. A. Sugden et al., 1987) (Fig. 1.4). The rise in intracellular Ca^{2+} occurs through an influx that is stimulated by α_1-adrenergic receptors. In addition, PLC is activated directly by a G-protein that acts through α_1-adrenergic receptors (Ho, Chik, & Klein et al., 1988b) (Fig. 1.4). Together, intracellular Ca^{2+} and diacylglycerol activate PKC.

PKC phosphorylates both the Gα subunit, which activates adenylyl cyclase as well as adenylyl cyclase itself (Fig. 1.4). When the G-protein is bound to adenylyl

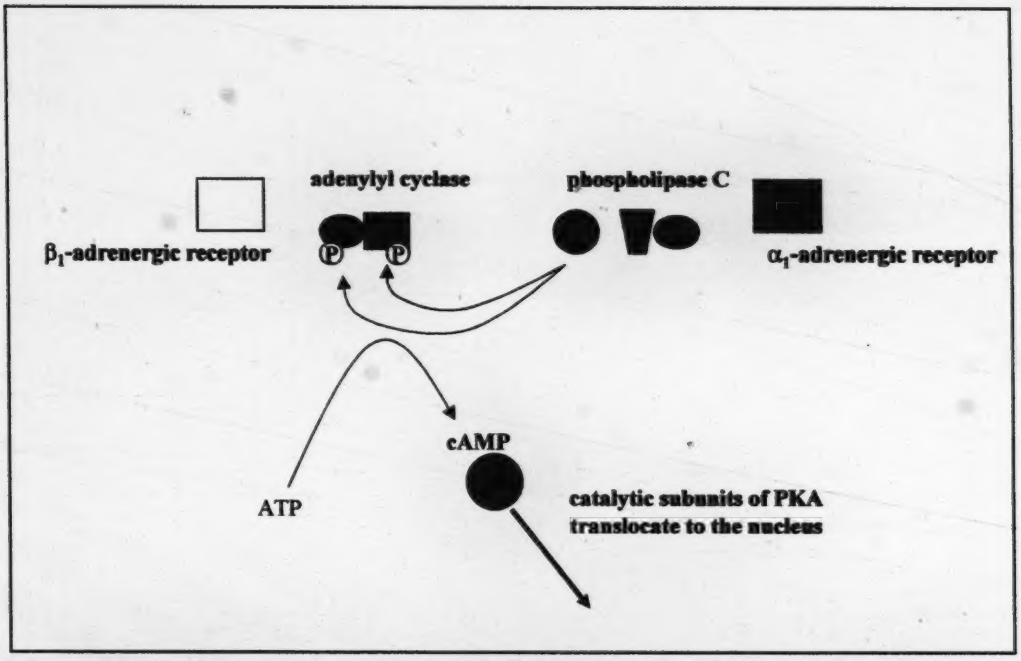

Figure 1.4 Potentiation of cAMP production. Once activated, PKC phosphorylates both adenylyl cyclase and the G-protein associated with β_1-adrenergic receptors. Phosphorylation results in the prolonged activation of these two proteins and the potentiation of cAMP production. cAMP binds to the regulatory subunits of PKA which causes a conformational change in the protein and the release of its catalytic subunits. The catalytic subunits of PKA can then translocate to the nucleus.

cyclase, GTP is converted to GDP, the Gα subunit changes its conformation and dissociates from adenylyl cyclase. Phosphorylation of the Gα subunit decreases the catalytic activity of the GTPase and slows down the conversion of GTP to GDP. By decreasing the GTPase activity, the Gα subunit is able to stimulate adenylyl cyclase for a longer period of time, resulting in the potentiation of the cAMP production. In addition, phosphorylation of adenylyl cyclase increases the duration of its activation, which again results in the potentiation of the cAMP response.

Together, the stimulation of both the α_1- and β_1-adrenergic receptors by NE results in a 4- to 10-fold increase in intracellular cAMP levels (Price et al., 2004; Price, Chik, & Ho, 2004). This rise in cAMP is essential for activating the cAMP-dependent protein kinase (PKA), which in turn acts through other mediators to initiate the transcription of *aa-nat* (Baler, Covington, & Klein, 1997; Klein & Berg, 1970; Klein, Berg, & Weller, 1970; Roseboom & Klein, 1995; Roseboom et al., 1996) (Fig. 1.4).

1.4 The role of cAMP response element binding protein (CREB) phosphorylation in *aa-nat* transcription

The increase of cAMP levels results in the activation of PKA (Gonzalez & Montminy, 1989) (Fig. 1.4). PKA resides in the cytoplasm as an inactive heterotetrameric protein made up of two regulatory subunits and two catalytic subunits. Two molecules of cAMP bind to each regulatory subunit and cause a conformational shift, resulting in the liberation of the catalytic subunits. Once released, the catalytic subunits passively diffuse into the nucleus and induce the transcription of *aa-nat* by phosphorylating CREB at serine 133 (Gonzalez & Montminy, 1989; Roseboom & Klein, 1995) (Fig. 1.5).

The amount of activated PKA catalytic subunits is directly proportional to the amount of CREB phosphorylation and gene transcription (Hagiwara et al., 1993). CREB is able to activate transcription by initially binding as a dimer to a conserved cAMP response element (CRE), TGACGTCA (Comb, Birnberg, Seasholtz, Herbert, & Goodman, 1986; Montminy, Sevarino, Wagner, Mandel, & Goodman, 1986). Phorphorylation of CREB allows the recruitment of an important co-activator, the 256kDa CREB binding protein (CBP) (Chrivia et al., 1993; Kwok et al., 1994; McManus & Hendzel, 2001) (Fig. 1.5).

The interaction between pCREB and CBP plays an important role in stimulating *aa-nat* transcription. At one point it was believed that CBP only acted as a scaffolding protein for other transcription factors because of its large size and its multiple protein binding domains (McManus & Hendzel, 2001). However, CBP possesses the ability to acetylate histones and other transcription factors such as TFIIE and TFIIF (Bannister & Kouzarides, 1996; Martinez-Balbas et al., 1998). The modification of histones is important for initiating gene transcription (Brownell et al., 1996). DNA, which is negatively charged, is wrapped around positively charged histone proteins. Transcription factors do not have access to the DNA when it is wound around the histones, so an acetyltransferase enzyme is responsible for acetylating the positive charges on the histone proteins to relax the conformation of the DNA. After acetylation of the histones, the DNA becomes loosely bound and transcription factors are able to interact with the DNA and initiate transcription (Brownell et al., 1996).

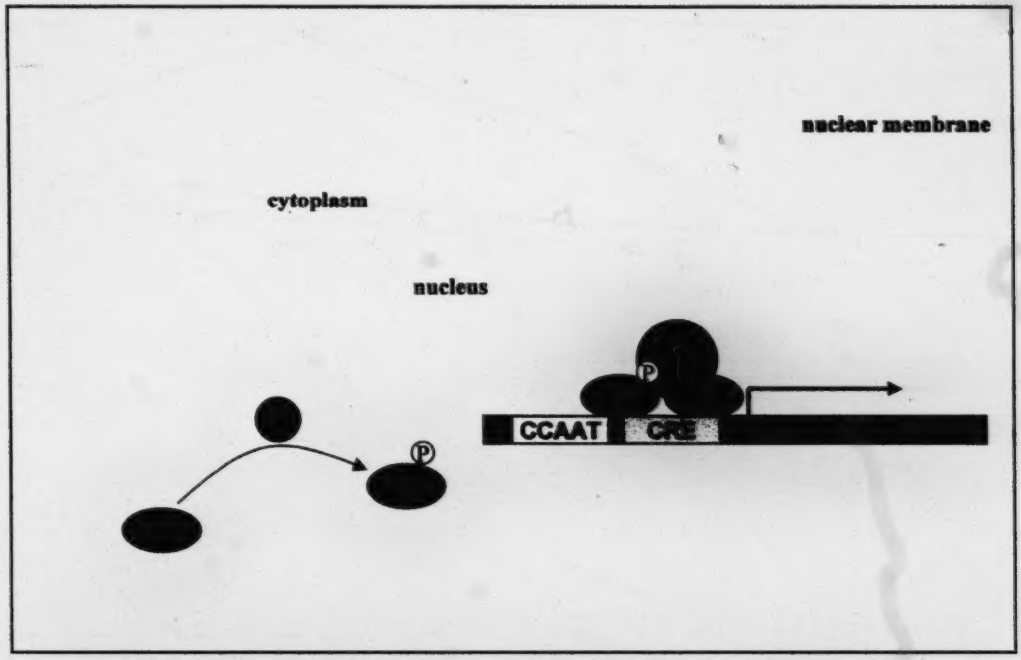

Figure 1.5 The role of CREB in *aa-nat* transcription . PKA translocates into the nucleus and phosphorylates cAMP response element binding protein (CREB). Once CREB is phosphorylated it can bind to the cAMP response element (CRE) in the *aa-nat* promoter and recruit the transcription factor CREB binding protein (CBP). CBP is a large protein which can recruit additional transcription factors including RNA polymerase II (Pol II).

1.5 Mechanism of CREB activated transcription

CREB activates transcription through two of its functional domains (B. Mayr & Montminy, 2001). The first domain (Q2: amino acids 160-283) is constitutively active, while the second domain (KID, kinase inducible domain: amino acids 100-160) is activated by phosphorylation of Ser 133. The Q2 domain helps to promote target gene expression through its interaction with the TFIID transcription complex (Nakajima, Uchida, Anderson, Parvin, & Montminy, 1997). Functional studies have revealed that the Q2 domain is necessary for transcriptional activation. In fact, without the domain, CREB acts as a repressor (Brindle, Nakajima, & Montminy, 1995; Felinski & Quinn, 1999). Recent studies suggest that the Q2 domain might help to assemble the RNA polymerase II complex by promoting the complex's formation (Felinski, Kim, Lu, & Quinn, 2001).

In contrast to the constitutively active Q2 domain, the KID domain is only activated though phosphorylation of Ser 133 (Hagiwara et al., 1993). This was demonstrated by mutagenesis studies whereby replacement of Ser 133 for Ala abolished CREB transcriptional activation capabilities (Gonzalez & Montminy, 1989). Phosphorylation of Ser 133 activates CREB activity by promoting the recruitment of CBP (Chrivia et al., 1993). In turn, the recruitment of CBP facilitates the activation of the RNA polymerase II complex (Kee, Arias, & Montminy, 1996; Nakajima et al., 1997) (Fig. 1.5). This result suggests cooperation between the Q2 domain and CBP. The Q2 domain helps to promote the assembly of the RNA polymerase II complex, while CBP facilitates its activation.

It is interesting to note that non-cAMP signals have the ability to phosphorylate CREB, yet they are unable to induce the activation of target genes through CREB (Sun,

Enslen, Myung, & Maurer, 1994). CREB phosphorylation alone is not sufficient to activate the transcription of cAMP regulated genes, including *aa-nat* (Spessert et al., 2000). The recruitment of CBP appears to be a very important factor for discriminating between cAMP induced and non-cAMP induced signals (B. M. Mayr, Canettieri, & Montminy, 2001; Wagner, Bauer, Schutz, & Montminy, 2000). For example, activation of PKC using the specific PKC activator, PMA, results in the phosphorylation of CREB. However, phosphorylation of CREB was not sufficient to recruit CBP and activate gene transcription. One explanation for this result is that either CREB or CBP is modified at additional sites in response to non-cAMP signals and that these modifications disrupt the formation of the CREB/CBP complex (B. Mayr & Montminy, 2001). Within the KID domain of CREB, there are two conserved lysine residues, which could be the site of acetylation, ubiquitination, or methylation. Although these modifications have not been demonstrated *in vivo*, it is possible that they could be a factor in discriminating between cAMP and non-cAMP signals. A potential mechanism for the discrimination between signals could be that these modifications are removed by a factor that is activated via PKA. Failure to remove these modifications would result in the inability to activate gene transcription.

Current evidence favours another model for the regulation of CREB activated transcription (Ernst, Wang, Huang, Goodman, & Korsmeyer, 2001; B. M. Mayr et al., 2001). This model suggests that an inhibitor, which binds to the KID domain of CREB, may block the association between CREB and CBP. Although the identity of this inhibitor is unknown, it may play a very important role in regulating CREB activated transcription. In particular, this inhibitor may help regulate the transcription of *aa-nat*. It

is necessary that the transcription of *aa-nat* be tightly regulated so that it is only expressed at night in response to NE → cAMP stimulation. This inhibitor could play a key role in helping to ensure that *aa-nat* is expressed at the appropriate times and by the proper signal.

The differential regulation of the 105 genes containing CREs is achieved by variations in its sequence and its location within the promoter. A half-site of CRE (CGTCA) exists in many promoters, yet it is less active than the full length palindrome (TGACGTCA) (Fink et al., 1988; Yamamoto, Gonzalez, Biggs, & Montminy, 1988). Variations in the location of the CRE site within the promoter and in relation to the TATA box, an AT-rich DNA sequence found in most eukaryotic promoters, plays a role in controlling the activity of the CRE site. Normally found within 100bp of the TATA box, the activity of the CRE site drops off rapidly as it is moved further upstream of the promoter (Tinti et al., 1997).

There is conflicting evidence as to whether phosphorylation of CREB actually increases its binding affinity for CRE sites (Nichols et al., 1992). *In vitro* binding assays indicate that phosphorylation of CREB on Ser 133 does not enhance the binding affinity of CREB for full palindromic CRE sites. However, phosphorylation does increase the binding affinity of CREB to CRE half-sites. This result is interesting because it suggests a different method of regulation for genes with full palindromic CRE sites and half-sites.

Ultimately, the phosphorylation of CREB results in the initiation of *aa-nat* transcription, causing a 150-fold increase in *aa-nat* mRNA and AA-NAT protein after 5 to 6 hours of NE stimulation (Roseboom & Klein, 1995). The amount of AA-NAT protein appears to be directly related to the level of *aa-nat* mRNA, suggesting that there

is little translational regulation of *aa-nat* mRNA. However, post-translational regulation of AA-NAT protein is a tightly controlled process and plays a very important role in modifying enzyme activity and melatonin production.

1.6 Post-translational regulation of AA-NAT

AA-NAT protein levels and activity are regulated by several factors. As mentioned previously, in response to NE stimulation at night, there is a significant induction of *aa-nat* transcription and in turn a 10- to 100-fold increase in AA-NAT protein levels (Klein & Berg, 1970; Klein et al., 1970). It is important to note that not only does PKA help initiate *aa-nat* transcription, it is also phosphorylates AA-NAT protein (Ganguly et al., 2001) (Fig. 1.6). Phosphorylation plays a very important role in helping AA-NAT bind to 14-3-3, a highly conserved scaffolding protein that exists as a dimer (Roseboom et al., 1994). 14-3-3 dimers preferentially bind the phosphorylated form of AA-NAT (Fig. 1.6). This association is essential for the nightly production of melatonin since it serves to protect AA-NAT from proteolytic degradation as well as to increase the enzymatic activity of the protein. Phosphorylation of Thr31 in the N-terminal region of AA-NAT was shown to increase its activity by 3-fold and to protect the enzyme from degradation by the proteasome, both *in vitro* and *in vivo* (Ganguly et al., 2001; Zheng et al., 2003). These results are important because they implicate cAMP/PKA not only in the initiation of transcription of the *aa-nat* gene, but also in the protection of the enzyme from degradation, and in modulation of enzyme activity.

More recently, it was shown that dual phosphorylation of AA-NAT is required for binding to 14-3-3 (Ganguly et al., 2005). Previously, it had been suggested that the C-

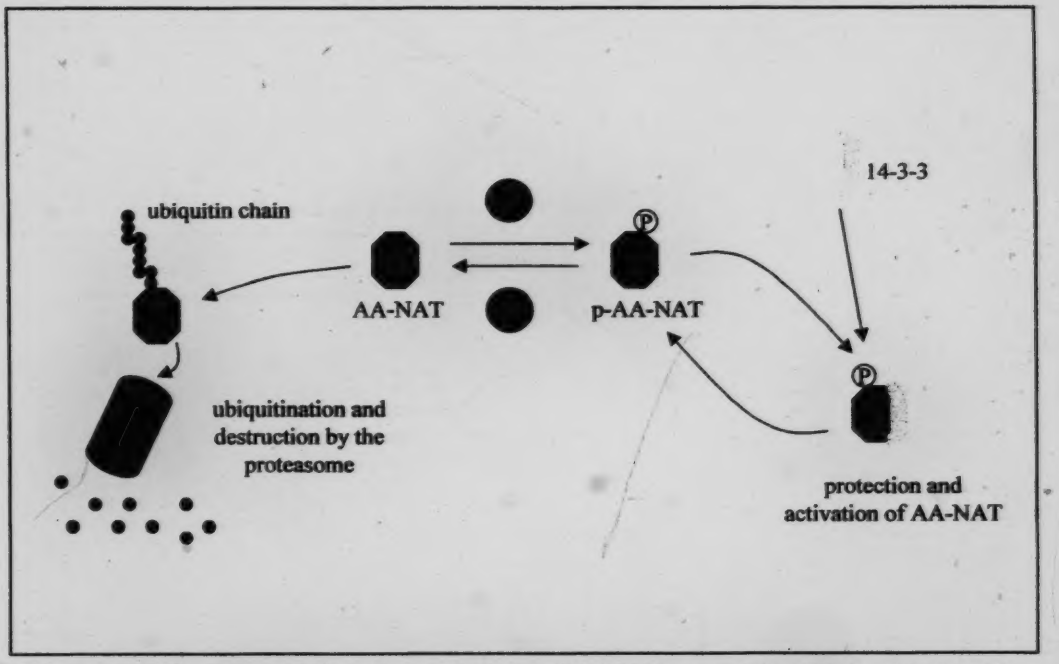

Figure 1.6 Post-translational regulation of AA-NAT protein. PKA also has the ability to phosphorylate AA-NAT protein which allows it to interact with 14-3-3, a scaffolding protein. Its association with 14-3-3 protects the enzyme from degradation by the proteasome and helps to increase its enzymatic activity. However, if AA-NAT dissociates from 14-3-3 it is prone to dephosphorylation, by a phosphoprotein phosphatase (PP), and subsequent ubiquitination, which targets the protein for destruction.

terminal region of AA-NAT remains unphosphorylated because it does not contain a consensus 14-3-3 binding motif (Aitken, 2003; Yaffe, 2004). However, it is now clear that phosphorylation of Ser205, in the C-terminal region of AA-NAT, is important for binding to 14-3-3. As expected, phosphorylation of Ser205 is also mediated by PKA.

In response to light, there is a rapid decrease in AA-NAT activity and melatonin production (Klein & Weller, 1972). In a study where rats were exposed to light after a prolonged period of darkness, it was shown that AA-NAT activity decreased significantly immediately after exposure to light. It was so rapid, that AA-NAT activity was virtually absent after 10 minutes. Later, it was demonstrated that this rapid reduction in enzyme activity was due to the selective degradation of AA-NAT protein by the ubiquitin-proteasome pathway (Gastel, Roseboom, Rinaldi, Weller, & Klein, 1998) (Fig. 1.6). Use of the β-adrenergic receptor blocker, L-propranolol, caused a rapid reduction in AA-NAT protein levels and activity. However, this reduction was abolished by the addition of dibutyryl cAMP, a cell permeable cAMP analogue, or the addition of lactacystin, a selective proteasome inhibitor. This study was further supported by data that suggest phosphodiesterase inhibitors also abolish the rapid reduction in AA-NAT protein levels when the pinealocytes were treated with L-propranolol (Klein, Buda, Kapoor, & Krishna, 1978). These results suggest that an abrupt reduction in cAMP levels may be responsible for the selective degradation of AA-NAT protein by the proteasome.

The current model for rapid degradation of AA-NAT by the proteasome proposes that when AA-NAT is not bound to 14-3-3, it can be rapidly dephosphorylated and ubiquitinated by a series of enzymes (Fig. 1.6). The addition of a long chain of ubiquitin molecules to a substrate targets the protein for degradation by the proteasome. In

contrast, when intracellular levels of cAMP are high, PKA will phosphorylate AA-NAT, which will allow it to associate with 14-3-3 (Fig. 1.6). As mentioned previously, this association protects AA-NAT from ubiquitination and subsequent degradation via the proteasome. However, most biological processes exist in equilibrium and the phosphorylation of AA-NAT is no exception. Small amounts of phosphorylated AA-NAT will naturally dissociate from 14-3-3. While unbound, AA-NAT can be dephosphorylated by a phosphoprotein phosphatase, which leaves the enzyme prone to ubiquitination (Fig. 1.6). In the absence of activated PKA, there will be a net dissociation of AA-NAT from 14-3-3 followed by dephosphorylation of the enzyme. This model is supported by evidence that there is a net dephosphorylation of AA-NAT at both the N- and C-terminal ends in response to light (Ganguly et al., 2005).

1.7 Repressor proteins involved in regulating *aa-nat* transcription

1.7.1 Inducible cAMP Early Repressor (ICER)

CREB belongs to a family of transcription factors that are all involved in regulating the transcription of CRE associated genes (Foulkes, Borrelli, & Sassone-Corsi, 1991; Hoeffler, Meyer, Yun, Jameson, & Habener, 1988). One member of this family is cAMP response element modulator (CREM). Unlike CREB, which is constitutively expressed in the pineal gland, CREM is induced in response to NE stimulation in the pineal gland (Foulkes, Duval, & Sassone-Corsi, 1996). Both CREB and CREM genes give rise to alternatively spliced products with distinctive activator or repressor properties (Foulkes & Sassone-Corsi, 1992; Walker, Girardet, & Habener, 1996).

One repressor in particular, which may play an important role in the repression of adrenergically induced *aa-nat* transcription, is inducible cAMP early repressor (ICER) (Foulkes, Borjigin, Snyder, & Sassone-Corsi, 1996). The *icer* gene is transcribed from an alternative intronic promoter from within the 3' end of the *crem* gene. Essentially, ICER is a truncated version of CREM, which lacks the activation domains, Q2 and KID, but contains the DNA binding region (bZIP). The absence of these activation domains is significant because it turns ICER into a powerful repressor of cAMP stimulated transcription. The bZIP region allows ICER to bind to CRE sites as a homo or heterodimer, yet it is unable to interact with CBP. This means that ICER and CREB compete for the same DNA binding domains and that a balance between the activator, pCREB, and the repressor, ICER, may important in determining whether *aa-nat* is transcribed. ICER is such a potent repressor that even very low levels may exert a substantial inhibitory effect on pineal melatonin synthesis (Pfeffer, Maronde, Molina, Korf, & Stehle, 1999). Also, immunohistochemical detection of basal levels of ICER supports the view that this repressor may set the threshold for induction of cAMP stimulated genes and prevent inadequate activation (Staiger et al., 2000; Stehle, von Gall, & Korf, 2001).

In response to NE stimulation at the onset of darkness, there is a significant up regulation of *icer* transcription in the rat pineal gland (Stehle, Foulkes, Molina, Simonneaux, Pevet, & Sassone-Corsi, 1993b). The peak of *icer* expression occurs in the second half of the night and precedes the down regulation of *aa-nat* mRNA and the decline in melatonin synthesis (Takahashi, 1994). In mice that are homozygous for a null-mutation at the CREM locus, whereby all CREM isoforms including ICER are

inactivated, *aa-nat* expression was elevated at all time points during the night (Foulkes et al., 1996). Not only were mRNA levels elevated, but *aa-nat* induction also occurred earlier in the night and persisted for a longer period than in wild-type mice. These results clearly suggest that ICER plays a significant role in negatively regulating *aa-nat* expression. However, *aa-nat* still displayed a diurnal rhythm indicating that ICER is likely not the only factor regulating *aa-nat* transcription. In addition to controlling *aa-nat* transcription, ICER also regulates its own expression through a negative feedback loop (Molina, Foulkes, Lalli, & Sassone-Corsi, 1993). As ICER levels accumulate in the nucleus it represses its own transcription by binding to four CRE sites within its promoter.

Since ICER is in direct competition with CREB for CRE sites, its activity is primarily determined by its intracellular concentration. In addition to the rate of transcription, a critical factor in regulating ICER protein levels is its degradation via the ubiquitin-proteasome pathway (Desdouets et al., 1995; Folco & Koren, 1997; Monaco, Foulkes, & Sassone-Corsi, 1995). Studies demonstrated that the use of a potent and highly specific proteasome inhibitor, lactacystin, resulted in increased levels of ICER protein. In addition, ICER was shown to be ubiquitinated *in vivo* and that in the presence of lactacystin, ubiquitinated ICER levels increased. Together, these results provide strong evidence that ICER protein levels are directly regulated by the proteasome. This is important since it demonstrates that the proteasome helps control the levels of a transcription factor that may directly inhibit *aa-nat* transcription. Because of these results, it will be important to investigate whether the proteasome plays a role in regulating the adrenergic induced transcription of *aa-nat*.

1.7.2 Downstream Regulatory Element Antagonist Modulator (DREAM)

Recently, it was suggested that another transcription factor called downstream regulatory element antagonist modulator (DREAM) might play a role in repressing cAMP-induced *aa-nat* transcription (Link et al., 2004). DREAM is a Ca^{2+}-regulated transcriptional repressor that binds to downstream regulator element (DRE) sites (GAGTCAAGG) as a homotetramer and represses transcription (Osawa et al., 2001; Osawa et al., 2005). DRE is a silencer element that does not require a specific orientation to suppress transcription (Carrion, Mellstrom, & Naranjo, 1998; Carrion, Mellstrom, Luckman, & Naranjo, 1998). However, for this element to be functional, it is important that it is placed in close proximity to the TATA box, an AT-rich DNA element essential for RNA polymerase II transcription (Carrion, Link, Ledo, Mellstrom, & Naranjo, 1999; Osawa et al., 2001). The close proximity to the TATA box suggests that DREAM interacts with other transcriptional machinery to suppress transcription.

DREAM has four Ca^{2+}- binding sites, all of which are essential for its function. Mutations in any one of these sites results in the constitutive repression target genes (Carrion et al., 1999). Interestingly, DREAM has been shown to block the recruitment of CBP by CREB through its interactions with CREB (Ledo, Carrion, Link, Mellstrom, & Naranjo, 2000; Ledo, Kremer, Mellstrom, & Naranjo, 2002). Increased levels of intracellular Ca^{2+} result in the disassociation of DREAM from DRE sites and the subsequent recruitment of CBP by phosphorylated CREB. This interaction between the CREB and CBP represents a point of cross talk between cAMP and Ca^{2+}-dependent signal cascades at the level of gene expression. Previously it was mentioned that CREB phosphorylation alone is not sufficient to activate *aa-nat* gene transcription (Spessert et

al., 2000). DREAM may be an additional repressor in *aa-nat* transcription, which prevents CREB phosphorylation alone from activating transcription (Ledo et al., 2002). Further evidence supports the hypothesis that DREAM could be a repressor of the *aa-nat* gene. A search for DRE sites in the promoter region of the rat *aa-nat* gene uncovered two direct DRE sites and two inverted DRE sites within close proximity to the transcriptional start site (Link et al., 2004). In addition, DREAM is highly expressed in the pineal gland and has the ability to repress transcription from reporter genes driven by the *aa-nat* promoter. *In vivo*, binding of the DREAM tetramer is controlled by the circadian clock (Link et al., 2004). In response to NE stimulation, DREAM tetramers bind with a much lower affinity to DRE sites than during the day when the pineal gland is not being stimulated. It is interesting to note that the increased binding of DREAM to the *aa-nat* promoter does not correspond to increased levels of DREAM mRNA or protein levels. There are no changes in DREAM mRNA or protein levels in response to NE stimulation. This suggests that the diurnal rhythm of DREAM binding activity may be regulated at a post-translational level. One possibility is that the formation of the DREAM tetramer, which binds to DRE sites, is regulated through post-translational modifications.

It is possible that phosphorylation of DREAM plays a role in the formation of the tetramer, yet this has not been examined. Secondly, levels of DREAM tetramer could be regulated by the ubiquitin-proteasome pathway. It is plausible that rapid degradation of the DREAM tetramer could be a mechanism for the derepression of *aa-nat* transcription in response to NE stimulatoin. As indicated previously, the activity of another protein repressor, ICER, is regulated by the proteasome. Although it has not been investigated, the activity of DREAM could also prove to be regulated in a similar fashion. If that were

the case, it would further suggest that the proteasome is an important regulator in adrenergic induced *aa-nat* transcription.

1.7.3 Fos-related antigen 2 (Fra-2)

Indirect evidence suggests that Fos-related antigen 2 (Fra-2), a member of the Fos family of transcription factors, may be involved in regulating *aa-nat* transcription (Baler & Klein, 1995; Foletta, 1996; Guillaumond et al., 2000; Nishina, Sato, Suzuki, Sato, & Iba, 1990). Members of the Fra-2 family control gene expression through their interaction with the activator protein-1 (AP-1) DNA consensus sites (Quinn, Takimoto, Iadarola, Holbrook, & Levens, 1989). In addition, they can also form heterodimers with CREB family members, suggesting a role for Fra-2 in cAMP induced transcription (Hai & Curran, 1991). Previous studies have shown that Fra-2 is under circadian regulation in the rat pineal (Baler & Klein, 1995). The authors suggest that Fra-2 may help to repress adrenergically induced genes, such as *aa-nat*, which contain the AP-1 element. If this is the case then both ICER and Fra-2 could work synergistically to repress the transcription of genes containing CRE and AP-1 sites. In addition, Fra-2 may be able to bind to both CRE (TGACGTCA) and AP-1 (TGAGTCA) sites given the similarities between the two elements (Sassone-Corsi, Ransone, & Verma, 1990). If this proves to be the case then Fra-2 may act as a general regulator of transcription in the pinealocytes. Two other studies confirmed the role of Fra-2 in regulating transcription, since it was found to be a major component of the AP-1 binding complex and it is responsible for the increased binding activity at AP-1 sites during the night (Guillaumond et al., 2000; Guillaumond, Becquet, Bosler, & Francois-Bellan, 2002).

In contrast to the results of the above studies, another study suggested that Fra-2 may not actually repress adrenergic induced *aa-nat* transcription (Smith et al., 2001). In the study by Smith (2001), a dominant negative Fra-2 mutant was created and then gene expression in the rat pineal was assayed. They found that *aa-nat* transcription was not affected by Fra-2 knockdown, even though the *aa-nat* promoter contains putative AP-1 sites. However, the expression of another adrenergically induced gene, 5'deiodinase II, was found to be increased when Fra-2 activity was knocked down.

It is interesting to note that Fra-2 can act as an enhancer of its own transcription. The circadian transcription of Fra-2 may be mediated by an auto-regulatory loop. Truncation of Fra-2 at amino acid 207 creates a dominant negative mutant and prevents the stimulation of the *fra-2* gene (Smith et al., 2001). In addition, when Fra-2 protein is phosphorylated it can induce *fra-2* gene expression *in vitro* (Murakami et al., 1997). As levels of Fra-2 protein build up in the nucleus, it will further increase its own transcription.

Studies to date provide conflicting evidence that Fra-2 acts as a repressor of adrenergically induced genes in the pineal. It appears that Fra-2 may only modulate the transcriptional activity of a few select genes such as 5'deiodinase II. However, it is possible, that since Fra-2 belongs to such a large family of related proteins that another member of the family may be compensating for the loss of Fra-2 in the knockdown experiments. Further investigations are needed to determine whether Fra-2 regulates *aa-nat* transcription.

1.8 The Ubiquitin-Proteasome Pathway regulates many biological processes

The ubiquitin-proteasome pathway plays a very important role in a wide variety of cellular processes. Among these are regulation of the cell cycle, apoptosis, cell differentiation, modulation of cell surface receptors, degradation of misfolded proteins, DNA repair, the inflammatory response, the biogenesis of organelles and transcription (Ciechanover, Orian, & Schwartz, 2000; Yang & Yu, 2003). In addition, this system has recently been shown to have an involvement in many neurodegenerative diseases such as the pathogenesis of Parkinson's, Alzheimer's, Huntington's, Prion diseases, and amyotrophic lateral sclerosis (Ciechanover & Brundin, 2003; Keller, Hanni, & Markesbery, 2000).

The 20S proteasome is a large, 700,000-dalton cylindrical shaped structure, which has multiple catalytic sites located within its hollow cavity (Baumeister, Walz, Zuhl, & Seemuller, 1998) (Fig. 1.7a). Access to these catalytic sites is tightly regulated to prevent the uncontrolled degradation of cellular proteins. As expected for such a large structure, the organization of the proteasome is very complex. In eukaryotic cells, there are 14 different genes that code for the different proteasome subunits (Heinemeyer, Trondle, Albrecht, & Wolf, 1994). Seven of these genes code for the α-type subunits, and the other seven genes code for the β-types subunits. The two outer rings of the proteasome consist entirely of α-type subunits, while the two inner rings consist entirely of β-type subunits (Fig. 1.7a). The structure of the proteasome suggests that it is a hollow tube with access to the centre via small openings at either end. However, studies indicate that the N-terminal portions of the outer α-rings control the access to the catalytic centre, much

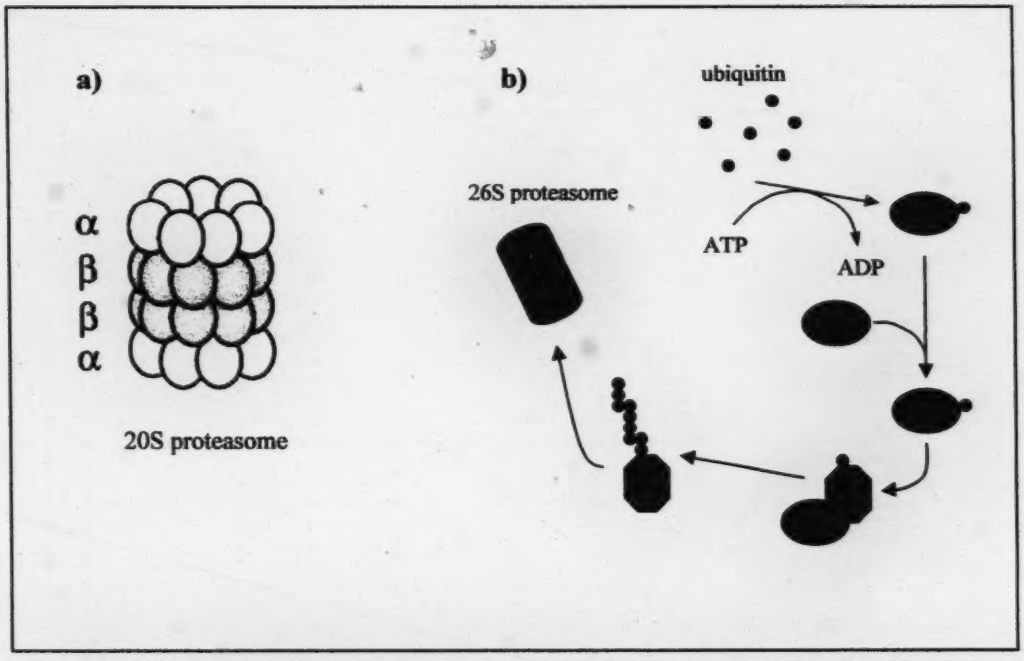

Figure 1.7 The ubiquitin-proteasome pathway. a) Structure of the 20S proteasome. It consists of 14 α subunits which make up the outer rings and 14 β subunits which make up the inner rings. The centre of the proteasome is a hollow cavity that contains the protease regions for degrading proteins. b) The ubiquitination process. Ubiquitin is modified into an activated form by the ubiquitin activating enzyme (E1) using energy from ATP. It is then transferred to a ubiquitin conjugating enzyme (E2) which ubiquitinates the substrate with the help of a ubiquitin ligase (E3). Polyubiquitinated substrates are then recognized by the 26S (20S + 19S cap) proteasome and degraded.

like a gated ion channel (Groll et al., 1997). Conformational changes in the proteasome induce the opening of the gates and allow access of the substrates to the catalytic centre.

The 20S proteasome alone is unable to degrade cellular proteins. Instead, proteolysis requires another large protein complex called the 19S regulatory particle (Glickman et al., 1998; Glickman, Rubin, Fried, & Finley, 1998). Together, these complexes make up the active 26S proteasome, a large 2100 kDa structure (Arrigo, Tanaka, Goldberg, & Welch, 1988). Binding of the 19S particle to either or both ends of the proteasome significantly enhances the ability of the proteasome to degrade ubiquitinated proteins. Degradation of substrates by the 26S proteasome requires continuous ATP hydrolysis (Hershko, Ciechanover, & Rose, 1979). Structural models of the proteasome suggest that the 19S particle helps to control access of the substrates to the active sites within the central cavity (Knowlton et al., 1997). Because folded proteins are too large to enter the central cavity, the regulatory particle is though to unfold the substrates in an ATP dependent manner and direct them toward the catalytic centre. In addition, the 19S particle is able to recognize polyubiquitinated substrates and therefore serves as a docking site for proteins destined to be destroyed.

Substrates are selected, targeted, and degraded by the proteasome via a multi-step process (Fig. 1.7b). The first step involves the covalent modification of a 76 amino acid protein called ubiquitin by the ubiquitin activating enzyme (E1) (Ciechanover, Heller, Katz-Etzion, & Hershko, 1981). As its name suggests, ubiquitin is expressed in all tissues. It serves an essential role in targeting proteins for degradation by the proteasome (Ciechanover, Elias, Heller, Ferber, & Hershko, 1980). E1 converts the C-terminal glycine of ubiquitin to a high energy thiol ester through the hydrolysis to ATP

(Ciechanover, Heller, Elias, Haas, & Hershko, 1980; Hershko, Ciechanover, Heller, Haas, & Rose, 1980; Hershko, Ciechanover, & Rose, 1981) (Fig. 1.7b). In the second step, one of several ubiquitin-conjugating enzymes (E2) transfers the activated ubiquitin protein to the substrate, which is bound to a ubiquitin ligase enzyme (E3). The transfer of ubiquitin to the substrate can either be direct or via an E3-ubiquitin thiol ester intermediate. The role of the ubiquitin ligase is to catalyze the transfer of the activated ubiquitin to an NH_2 group of an internal lysine residue (Breitschopf, Bengal, Ziv, Admon, & Ciechanover, 1998). There are no conserved sites for ubiquitination and therefore selection of the ubiquitin conjugation site is likely determined by the interaction between a substrate and its specific ubiquitin ligase. For a substrate to be targeted for degradation by the proteasome, it must be polyubiquitinated (Hershko & Heller, 1985). In other words, a long chain of ubiquitin molecules must be added to the substrate to trigger its degradation. By a series of successive reactions that were previously described, multiple ubiquitin proteins are attached to the internal Lys48 residue of the previously conjugated ubiquitin molecule (DeMartino & Slaughter, 1999). This chain then serves as a marker for the proteasome.

The structure of the ubiquitin system is hierarchal in the sense that there are more E3 enzymes than E2s than E1s (Ciechanover, Orian, & Schwartz, 2000). In fact, there is only a single E1 species, which activates all the ubiquitin required for conjugation to substrates. There are several species of E2 enzymes and each one can interact with multiple E3 enzymes. Although there have only been a number of E3 enzymes described so far, it appears that this is a rapidly growing family of proteins.

Why is it that some proteins are short-lived within the cell or why are certain proteins only degraded in response to an extracellular signal? A good example of the latter is the rapid degradation of AA-NAT protein in response to light. The hierarchal nature of the ubiquitin pathway allows for high specificity of protein degradation (Ciechanover, Orian, & Schwartz, 2000). Specificity is imparted by the vast number of E3 enzymes that recognize substrates. A substrate must be bound to an E3 enzyme prior to ubiquitination since E3 enzymes catalyze the conjugation of ubiquitin (Ciechanover, Orian, & Schwartz, 2000). Recognition is mediated by specific structural motifs within the substrates. Some substrates have these motifs encoded within the protein itself and are therefore continuously degraded, while many other proteins require modifications such as changes in their phosphorylation state, oligomerization, post-translational modifications, or association with another protein before they can be recognized by an E3 (Ciechanover & Brundin, 2003; Ciechanover, Orian, & Schwartz, 2000). Many transcription factors are regulated by the proteasome and must disassociate from the DNA before they can be ubiquitinated. In other cases, the E3 enzyme must be modified before it can bind to the substrate.

It is rare that only one E3 recognizes one substrate (Ciechanover & Brundin, 2003). Commonly, E3 enzymes recognize general motifs and are able to bind to multiple substrates. At this point it is uncertain which E3 enzyme recognizes AA-NAT and targets it for degradation. It is likely that dephosphorylation of AA-NAT and its dissociation from 14-3-3 allows it to be recognized by its corresponding E3. Binding with 14-3-3 protects the N-terminal region of AA-NAT, which contains the putative ubiquitination site. The identity of the E3 involved in targeting AA-NAT for destruction is of particular

interest since it plays such a vital role in the diurnal rhythm of AA-NAT activity and melatonin production.

1.9 The use of proteasome inhibitors to investigate biological processes

Recently, the development of specific pharmacological proteasome inhibitors has allowed researchers to look at the role of the proteasome in many cellular processes *in vivo* (Lee & Goldberg, 1998). For example, if the use of such an inhibitor blocks a decrease of a particular protein or actually increases the level of that protein then it strongly suggests that the proteasome is involved in its regulation. There are several types of low-molecular weight cell permeable inhibitors that are commonly used. The peptide aldehydes are a group of inhibitors that act as substrate analogues. Cbz-leu-leu-leucinal (MG132) is a member of this group and it binds reversibly to one of the catalytic sites causing inhibition of the chymotrypsin-like protease activity (Lee & Goldberg, 1996).

Another commonly used group of inhibitors, which are structurally unrelated to the peptide aldehydes, are the naturally occurring lactacystin derivatives. They were originally isolated from *Streptomyces* because of their ability to promote growth in cultured neurons as well as their ability to prevent cell division (Omura & Matsuzaki et al., 1991; Omura et al., 1991). Later it was discovered that lactacystin compounds act by inhibiting the proteasome. They act as pseudosubstrates that bind covalently to highly conserved amino-terminal threonine residues of the β-subunits (Fenteany et al., 1995). The covalent modification of the amino-terminal threonines by lactacystin has a drastic effect on proteasome activity since it irreversibly inhibits all three protease activities of the proteasome: the chymotrypsin-like, trypsin-like, and the postglutamyl-like activities.

Recent studies have used these drugs to investigate various processes such as the role of the proteasome in glucose stimulated (pro)insulin synthesis and secretion in β-cells (Kitiphongspattana, Mathews, Leiter, & Gaskins, 2005). Another study used MG132 to demonstrate that the proteasome is responsible for generating most of the peptides on MHC I molecules (Rock et al., 1994). As research progresses in the field of the proteasome, the use of these specific inhibitors will help to uncover the vital importance of the proteasome in a wide array of cellular processes.

Of particular interest is the role of the proteasome in the regulation of gene transcription. Changes in gene expression are essential in virtually all biological processes ranging from cell division to circadian rhythms. Recently, the use of proteasome inhibitors revealed that the proteasome can play an important role in regulating gene expression. One study looked at the effects of MG132 and clasto-lactacystin-β-lactone (c-lact) on the tumor marker gene, glutathione S-transferase P1 (GSP P1), a cellular defense gene that is highly expressed gene in hepatocellular carcinoma cells (Usami et al., 2005). Inhibition of the proteasome resulted in a significant increase in GST P1 transcription. This result suggests a potentially critical role for proteasome in the regulation of cellular defense genes such as GST P1. In contrast to the last study, treatment of adipocytes of either MG132 or lactacystin caused a significant reduction in the expression of GLUT4, a widely expressed glucose transporter (Cooke & Patel, 2005). Potentially, a repressor of GLUT4, which may be regulated by the proteasome, was allowed to accumulate and inhibit GLUT4 gene expression. Based on these studies, it is clear that proteasome activity can dramatically influence gene expression. Further research into the role of the proteasome in transcription will likely

prove to be very exciting since it will help scientists understand how a cellular protease can influence a vast array of biological processes by regulating gene expression.

1.10 Specific aim of the present study

In the rat pineal gland, the steady state level of AA-NAT protein, besides transcriptional and translational control, is also regulated by proteasome mediated degradation. At the end of the dark phase, AA-NAT is targeted for degradation by the proteasome. Although the effect of the proteasome on the steady state levels of AA-NAT protein have been well established, the potential role of the proteasome in modulating the transcription of the *aa-nat* gene has yet to be investigated. Based on previous studies in other cell types, it is clear that proteasome activity can dramatically influence gene expression.

With these findings in mind, the specific aim of the present study was to determine whether the transcription of *aa-nat* was regulated by the proteasome. Using the specific proteasome inhibitors, MG132 and c-lact, this study examined the consequences of blocking proteasomal proteolysis on adrenergic induced *aa-nat* transcription, AA-NAT protein levels, AA-NAT enzymatic activity and melatonin production.

Specifically, this study examined:

1) the time dependent effect of blocking proteasomal proteolysis on *aa-nat* transcription

2) whether the observed effects of MG132 or c-lact on *aa-nat* transcription were dependent on simultaneous NE stimulation

3) whether the effects of proteasome inhibitors on adrenergic induced transcription was long-lasting

4) the selective effect of MG132 and c-lact on *aa-nat* transcription

5) the site of action of proteasomal inhibitors on adrenergic-stimulated *aa-nat* transcription

6) whether a protein repressor is mediating the observed effects of proteasomal inhibitors on *aa-nat* expression

7) the identity of the protein repressor involved in the proteasomal inhibitor effects on NE-stimulated *aa-nat* transcription.

Not only will the results of this study help to understand the mechanisms that regulate the rhythmic transcription of *aa-nat*, but they will also contribute to the field of cAMP gene regulation. Like AA-NAT, a vast number of genes in a wide variety of cell types are regulated by cAMP dependent mechanisms. The data from this study may have general implications for other researchers studying cAMP mediated gene expression.

2. Materials and Methods

2.1 Materials

Norepinephrine, cycloheximide, and dibutyryl cAMP were obtained from Sigma Aldrich Co. (St. Louis, MO). MG132, c-lact and Suc-LLVY-AMC (N-succinyl-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin) were obtained from Biomol Co. (Plymouth Meeting, PA). [^{3}H] Acetyl-coenzyme (specific activity, 1 mCi/mmol), [^{125}I] cAMP (specific activity, 2000 Ci/mmol) was from Amersham Biosciences (Piscataway, NJ). [^{3}H] MT (specific activity, 81.1 Ci/mmol) was obtained from New England Nuclear (Boston, MA). Polyclonal anti-MKP-1 antibody and polyclonal anti-CREM antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal anti-glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) antibody was obtained from Ambion Inc. (Austin, TX). Monoclonal anti-DREAM antibody was obtained from Upstate Co. (Charlottesville, VA). Polyclonal anti-20S proteasome α/β subunits antibody was obtained from Biomol Co. (Plymouth Meeting, PA). Horse radish peroxidase (HRP)-linked goat anti-rabbit (GAR) antibody was obtained from Bio-Rad Laboratories, Inc. (Hercules, CA). HRP-linked goat anti-mouse (GAM) antibody was obtained from Chemicon Corp. (Temecula, CA). Polyclonal antibodies for the radioimmunoassay of melatonin were obtained from CIDTech Co (Mississauga, ON, Canada). Polyclonal anti-AA-NAT$_{25-200}$ (AB3314) antibody was a gift from Dr. D.C. Klein (National Institute of Child Health and Human Development, NIH, Bethesda, MD). Polyclonal antibodies for the radioimmunoassays of cAMP were gifts from Dr. A. Baukal (National Institute of Child Health and Human Development, NIH, Bethesda, MD). Papain dissociation system

was obtained from Worthington Biochemical Corporation (Lakewood, NJ). All other chemicals were of the purest grades available commercially.

2.2 Preparation of cultured pinealocytes and drug treatments

All procedures were reviewed and approved by the Health Sciences Animal Policy and Welfare Committee of the University of Alberta (Edmonton, Alberta). Sprague Dawley rats (male; weighing 150 g) were obtained from the University of Alberta Biological Sciences animal unit. Pinealocytes were prepared by papain dissociation of freshly dissected rat pineal gland (Buda & Klein, 1978). The glands were cleaned in a sterile Petri dish in ice cold sterile Dulbecco's modified Eagle's medium (DMEM) with Ca^{2+} /Mg^{2+} to remove any excess tissues. The glands were then transferred to a 15 ml sterile tube and washed several times in both DMEM with Ca^{2+}/$Mg^{2+}$and phosphate buffered saline (PBS), pH 7.2. Next, glands were treated with 0.005% DNase and 20 units/ml Papain in Earle's balanced salt solution (EBSS) at 37°C for 60 minutes under 95% air and 5% CO_2. During the incubation, the glands were vortexed briefly every 15 minutes. After 45 minutes, the glands were gently mixed using a 5ml sterile pipette. After the dissociation step, 1 mL of fetal calf serum (FCS) was added and the glands were centrifuged at 1300 x g for 10 minutes at room temperature. Glands were resuspended in DMEM + 10% FCS + 0.005% DNase and further dissociated by pipetting. Glands were centrifuged again at 1300 x g for 10 minutes at room temperature. Cells were resuspended in DMEM + 10% FCS and small tissue contaminants were removed by layering the cell suspension onto albumin ovomucoid inhibitor solution. The layered gradient was centrifuged at 500 x g for 8 minutes at room temperature. The cell

pellet was washed twice in DMEM containing 10% FCS and 1% penicillin, streptomycin, amphotericin (PSF) and resuspended in 10 ml of DMEM containing 10% FCS and 1% PSF. 40 µl of cells were mixed with 40 µl of trypan blue then they were counted using a hematocytometer. Cells were suspended in DMEM containing 10% FCS and 2% PSF and maintained before the experiment at 37°C for 24 hours in a mixture of 95% air and 5% CO_2. Cells (1.0×10^5 cells/200 µl DMEM containing 10% FCS and 1% PSF) were treated with different drugs in sterile microcentrifuge tubes for the time periods indicated. In the washout studies, cells were gently pelleted at 5000 x g for 2 minutes and resuspended in DMEM containing 10% fetal calf serum and drugs as indicated. For AA-NAT assay, 0.5×10^5 cells/200 µl DMEM containing 10% FCS and 1% PSF were used. For cAMP assay, 0.2×10^5 cells/200 µl DMEM containing 10% FCS and 1% PSF were used. For melatonin assay, the medium was collected from the pelleted cells and melatonin was extracted using methylene chloride. Treated cells were collected by centrifugation (2 min, 12000 x g). Samples for Western blot analysis were boiled for 5 min in 1x sample buffer and stored at 4°C until analysis. Samples for RNA isolation were immediately homogenized in Trizol (Invitrogen Co, Carlsbad, CA).

2.3 Western Blotting

Cell pellets were lysed in 1X sample buffer (equal volumes of 2X Sample Buffer and Buffer A which contains 20 mM Tris-HCl, 2 mM EDTA, 0.5 mM EGTA, 2 mM phenylmethylsulfonylfluoride, 1 µg/ml each of aprotinin, leupeptin, and pepstatin, 1 mM sodium vanadate, and 1 mM sodium fluoride, pH 7.5). Samples were then boiled for 5 minutes and placed at 4°C until electrophoresis. SDS–PAGE was performed according to

the procedure of Laemmli and described in previous studies ((Laemmli, 1970; Price, Chik, & Ho, 2004)) using 10% or 12% acrylamide and 1 mg/ml sodium dodecyl sulfate (Mini-Protein II gel system, Bio-Rad Laboratories, Inc., Hercules, CA). The SDS polyacrylamide gels were run at 170 V for approximately 1 hour in electrophoresis buffer (25 mM Tris – HCl, 190 mM glycine, 3.5 mM SDS). The gels were then placed in Towbin's buffer (25 mM Tris-HCl, 190 mM glycine, 20% methanol) and allowed to equilibrate for 20 minutes before the transfer was set up in a mini vertical gel system, E-C Apparatus Corporation (St. Petersburg, FL). The gels were transferred in Towbin's buffer onto polyvinylidene fluoride (PVDF) membranes, Millipore Corporation (Bedford, MA), for 1.5 hours at 45 V. The membranes were then blocked in 5 % skim milk in TTBS (20 mM Tris – HCl, 0.5 M NaCl, 0.005% Tween – 20 (Sigma Aldrich Co.)) for 1.5 hours and probed with primary antibody. The blots were probed overnight at 4°C by gentle rocking in TTBS containing 5% skim milk and primary antibody at the following dilutions: AA-NAT (1:2000), ICER (1:250), DREAM (1:2000), Fra-2 (1:500), GAPDH (1:20000), MKP-1 (1:133), 20S Proteasome α/β subunits (1:1000). Following the overnight incubation, the membranes were washed 4 times on an orbital shaker for 15 minutes in TTBS containing 5% skim milk. They were probed with HRP-linked secondary antibody for 1 hour in TTBS containing 5% skim milk at the following dilutions: GAR (1:2000), GAM (1: 4000). The membranes were washed on an orbital shaker for 2 hours in TTBS with at least four changes of the wash solution. Immediately prior to developing the membranes in ECL solution, the membranes were placed in TBS for 5 minutes. The membranes were developed in ECL Plus Solution (Amersham Biosciences (Piscataway, NJ) for one minute and exposed on X-ray film (Fuji Film Co,

Mississauga, Ontario).

2.4 RT-PCR

Total RNA was isolated from cultured pineal cells using Trizol (Invitogen Co., Carlsbad, CA). Cell pellets were homogenized in 600 µl of Trizol and stored overnight at 4°C until the RNA extraction was performed. 120 µl of chloroform (Fisher Scientific, Whitby, ON) was added to the homogenate and the tubes were shaken vigorously by hand for 15 seconds. The tubes were left at room temperature for 5 minutes then centrifuged at 12000 x g for 15 minutes at room temperature. The aqueous phase (325 µl) was transferred to a new tube containing 5 µl of glycogen (5 µg/µl) and the RNA was precipitated using 400 µl of isopropanol. RNA was left to precipitate for 10 minutes at room temperature then centrifuged at 12000 x g for 15 minutes at room temperature. The supernatant was removed by aspiration and the pellet was washed in 800 µl of 75% ethanol. Again, the RNA was centrifuged at 12000 x g for 7 minutes at room temperature. The ethanol was removed by aspiration and the pellets were dried on the bench for 5 minutes. The RNA was resuspended in 27 µl of RNase free water and incubated in a 55°C water bath for 10 minutes.

First strand cDNA was synthesized from the isolated RNA using an Omniscript reverse-transcriptase kit (Qiagen Inc., Valencia, CA) with an oligo-dT primer. Briefly, a master mix was made up containing (per tube): 1.5 µl 10X Reaction Buffer, 1.5 µl dNTPs, 1.5 µl oligo d-T primer, 0.75 µl RNase-OUT (Invitrogen Corp.) and 0.75 µl Reverse Transcriptase. 6 µl of the master mix was added to each tube then the tubes were

placed in a 37°C water bath for 1 hour. To terminate the reaction, the tubes were incubated in a 93°C water bath for 5 minutes. For PCR of cell extracts, 3 μl of a 1:10 dilution of cDNA was used. PCR was performed in a 29.3 μl reaction mixture containing 10 mM Tris, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 100 μM of each dNTP, 0.75 U Taq polymerase (Perkin–Elmer Cetus, Emeryville, CA) and 1 μM each of the two primers. PCR reactions were as follows: denaturing for 1 minute at 94°C, annealing for 1 minute at 63°C, and extension for 1 minute at 72°C. Initial denaturing and final extension were both 5 minute in duration. Cycle numbers varied slightly between cell preparations, but in general, 22 cycles were used to amplify *gapdh*, 25 cycles to amplify *mkp-1*, and 23 cycles to amplify *aa-nat*. All reaction sets included water blanks as negative controls. Amplified products were separated on ethidium bromide-stained 1.5% agarose gels. PCR products were confirmed by sequencing. All primer sequences were designed using rat gene cDNA sequences obtained from BLAST (Altschul et al., 1997) and primers were selected from 3' regions using the internet based primer design program, *Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www_slow.cgi)*. Primers used are as follows: *MKP-1*: left primer: 5'-CTG CTT TGA TCA ACG TCT CG-3'; right primer: 5'-AAG CTG AAG TTG GGG GAG AT-3'; *AA-NAT*: left primer: 5'-GGT TCA CTT TGG GAC AAG GA-3'; right primer: 5'-GTG GCA CCG TAA GGA ACA TT-3'; *GAPDH*: left primer: 5'-TGA TGA CAT CAA GAA GGT GG – 3'; right primer: 5'-TTT CTT ACT CCT TGG AGG CC-3'.

2.5 MT Assay

MT was extracted from the cell medium using methylene chloride as previously described ((Man, Rustaeus, Price, Chik, & Ho, 2004)). Briefly, 1 ml of methylene chloride was added to the cell medium and the tubes were vortexed vigorously for 30 seconds to extract the MT. The tubes were then centrifuged at 10000 x g for 30 seconds and the cell medium was removed by aspiration. 600 µl of the methylene chloride transferred to new microcentrifuge tubes and was evaporated overnight in the fume hood. The following day, MT was resuspended in 300 µl of MT buffer (50 mM Na_2HPO_4, 150 mM NaCl, 0.1% gelatin (w/v), and 250 µM thimerosol) by vortexing for 20 minutes. The assay was set up by preparing a standard curve using melatonin standard diluted in MT buffer ranging from 0 pg – 640 pg melatonin per 500 µl. 500 µl of each dilution of melatonin standard was added to tubes in duplicate to create the standard curve. The samples were prepared by adding 5 µl sample and 495 µl MT buffer to each tube. Melatonin antibody (100 µl; 1:650000) was added to each tube, except the totals and non-specific blanks. 50 µl of [^{3}H] MT (approx. 2500 cpm; specific activity, 81.1 Ci/mmol) was added to each tube. The tubes were vortexed and incubated overnight at 4°C. The following day, 650 µl of saturated ammonium sulphate and 25 µl of 1.75M calcium sulphate were added to each tube except the totals. The tubes were vortexed then incubated for 1 hour at 4°C to precipitate the antibody and bound melatonin. After, the tubes were vortexed and centrifuged at 3000 x g for 30 minutes at 4°C. The supernatant was decanted; the precipitate was resuspended in 500 µl of water, mixed with 1 ml of scintillation fluid and counted in a liquid scintillation counter by Canberra Packard

(Montreal, PQ). The assay was able to detect melatonin levels as low as 1pg. Melatonin production was expressed as pmol/10^5 cells.

2.6 Proteasome Activity Assay

The supernatant was removed and the pelleted pinealocytes were frozen in dry ice and stored overnight at –80°C. The following day they were lysed by sonication (three short pulses) in 40 µl of ice-cold lysis buffer (PBS, 1% Triton X-100, 1 mM DTT). The lysates were centrifuged for 10 minutes at 12,000 x g at 4°C and the supernatant was used for the assay. In a 96-well BD Optilux plate (BD Biosciences, Mississauga, ON), 40 µl of the crude cell lysate was combined with 10 µl of 10X assay buffer (250 mM HEPES, 5 mM EDTA, pH 7.6, 0.3% SDS), 40 µl water, 10 µl LLVY-AMC (Biomol Co.) to give a final concentration of 62.5 µM (Shibatani & Ward, 1995). A standard curve was prepared using a concentration range of 0 – 12.5 µM AMC (Sigma Aldrich Co.) The 96-well plate was placed in a 37°C incubator in a mixture of 95% air and 5% CO_2 for 2 hours. Fluorescence was measured using a Thermo Electron Fluorescent plate reader (380/460 nm filter set). Cell lysate background fluorescence readings were prepared by adding 50 µM c-lact immediately after the cells were lysed. The background readings were subtracted from all the sample readings to determine the proteasome activity. The proteasome activity was expressed as pmol of AMC produced/h/10^5 cells.

2.7 Cyclic Nucleotide Assay

Cells were collected by centrifugation at 12000 x g for 2 minutes. The supernatant was removed by aspiration and the cells were immediately frozen in dry ice. Once all the tubes had been collected, the cells were lysed in 100 µl of 5 mM acetic acid by boiling the samples for 5 minutes. The assay was set up by preparing a standard curve for cAMP in the concentration range of 0 – 1000 fmol/ 100 µl. 100 µl of each concentration was added to tubes in duplicate. The samples were prepared by adding 20 µl of each lysate to 5 ml borosilicate test tubes. To make the concentration of acetic acid in each tube identical, 100 µl of water was added to each of the sample tubes, 20 µl of 5 mM acetic acid was added to each tube in the standard curve of the cAMP assay. Since the antibody has a higher affinity for the acetylated forms of cAMP, the contents of the standard curve and the samples were acetylated. A mixture of 2:1 triethylamine : acetic anhydride was prepared and 10 µl was added to each tube. The tubes were immediately vortexed after the addition of the acetylation mixture. After, 1 µl of [^{125}I] cAMP (specific activity, 2000 Ci/mmol), 1 µl of cAMP antibody (1: 50000), 2 µl of normal rabbit serum, and 200 µl of sodium acetate buffer were added to each tube. The tubes were stored overnight at 4°C. The following day, sheep anti-rabbit antibody (1:10) was added to each tube and then they were incubated at 4°C for 1hour. 1 ml of polyethylene glycol was added just before centrifugation at 4°C for 30 minutes at 3000 x g. The supernatant was decanted and the tubes were counted in a gamma counter. cAMP levels were expressed pmol/10^5 cells

2.8 AA-NAT Enzymatic Assay

AA-NAT activity was determined using a radioenzymatic assay as described previously (Man et al., 2004). Treated pinealocytes were stored frozen in dry ice until homogenization in a reaction mixture of 0.1 M phosphate buffer (pH 6.8) containing 30 nmol [^{3}H] acetyl coenzyme A (specific activity, 1 mCi/mmol) and 1 μmol tryptamine hydrochloride in a final volume of 60 μl. Three short pulses of gentle sonication were used to homogenize the samples. Immediately after sonication, the samples were placed in an ice water bath then centrifuged for 30 seconds at 5000 x g. The reaction mixture was incubated at 37°C water bath with gentle agitation for 1 hour. At the end of the incubation period, the reaction was stopped by the addition of 1 ml methylene chloride. After vortexing for 30 seconds, the aqueous phase was removed, and the organic phase was washed three times with 0.1 M phosphate buffer (pH 6.8). The remainder of the organic phase was transferred to a scintillation vial and evaporated to dryness, and radioactive acetylated product was determined by scintillation counting. AA-NAT activity was expressed as nmol/h/10^5 cells.

2.9 Results and Statistical analysis

For quantitation of RT-PCR analyses, gel images were acquired with Kodak 1-D software on a Kodak 2000R imaging station (Eastman Kodak Co., Rochester, NY). For analyses of Western blots, exposed films were scanned and band densitometry of acquired images was performed with Kodak 1-D software. Densitometric values were normalized to % maximal and presented as the mean ± SEM from at least three independent experiments. For radioimmunoassay or radioenzymatic assays, data were

presented as the mean ± SEM from at least three independent experiments. Statistical analysis involved either a paired *t* test or ANOVA with the Newman-Keuls test. Statistical significance was set at $p < 0.05$.

3. Results

3.1 Time-dependent effects of proteasomes in adrenergic induction of *aa-nat* transcription

Treatment of cultured pinealocytes with NE (3 μM) for 6 hours caused a large increase in NE-stimulated *aa-nat* mRNA (Fig. 3.1a) and protein levels (Fig. 3.1b). Compared with cells stimulated with NE alone for 6 hours, exposure to a proteasome inhibitor, c-lact (10 μM), for the last 3 hours of NE treatment caused a further increase in AA-NAT protein (Fig. 3.1b) and enzyme activity (Fig. 3.1c) as reported previously (Gastel et al., 1998), but had no significant effect on the level of *aa-nat* mRNA (Fig. 3.1a). In contrast, treatment of pinealocytes with c-lact (10 μM) and NE for 3 hours followed by an additional 3 hours treatment with NE alone caused a reduction in the level of NE-stimulated *aa-nat* mRNA (Fig. 3.1a), protein (Fig. 3.1b) and enzyme activity (Fig. 3.1c). A similar inhibition of *aa-nat* transcription was observed when pinealocytes were treated with MG132 (3 μM), a structurally unrelated proteasome inhibitor to c-lact, for the first 3 hours of a 6 hour treatment with NE (Fig. 3.2). These results indicate that proteasomal inhibition can significantly reduce NE stimulated *aa-nat* transcription during the early phase of NE stimulation.

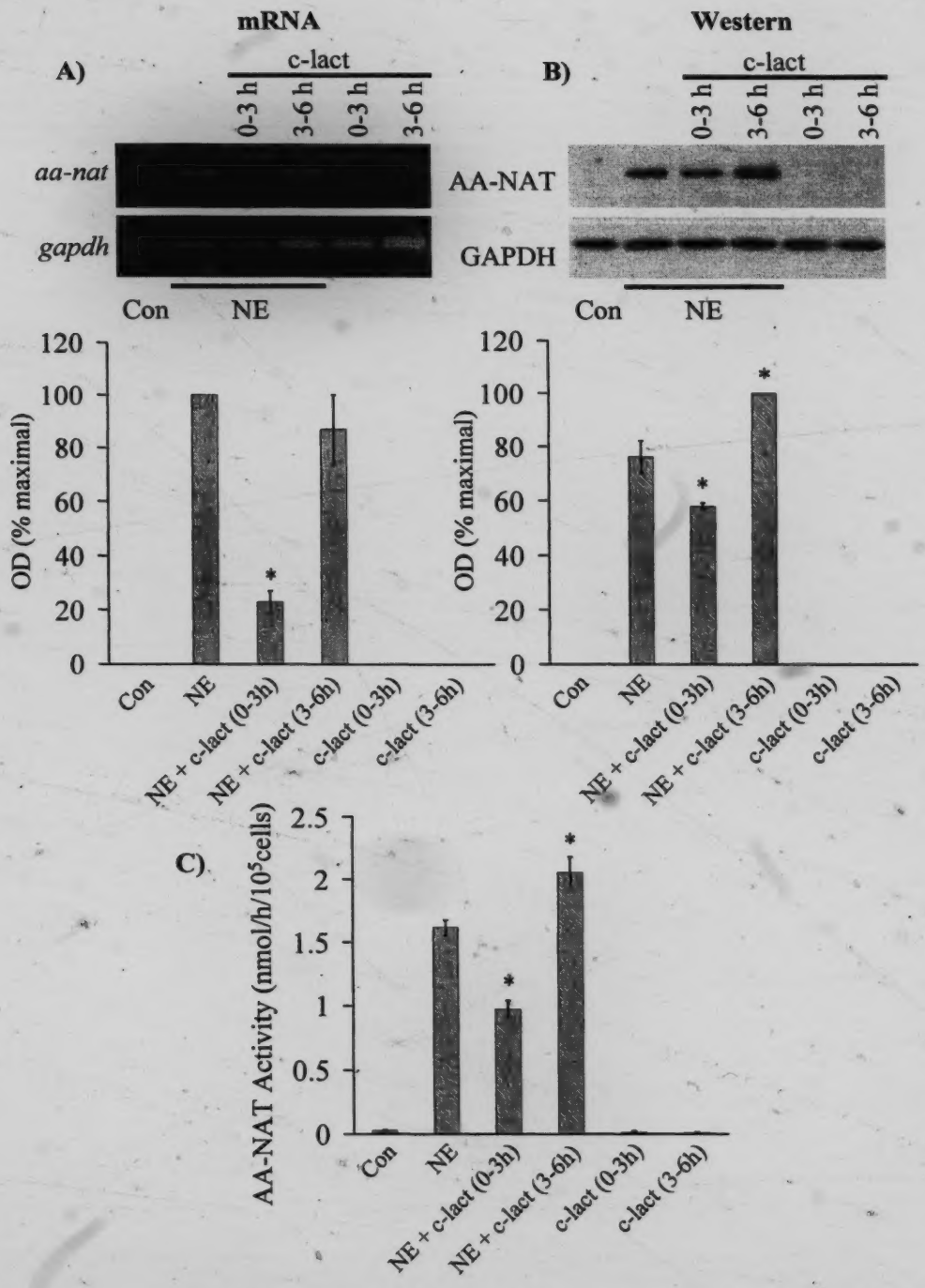

Figure 3.1. Time-dependent effects of c-lact on adrenergic induced *aa-nat* mRNA, protein and enzyme activity . (see next page for figure legend)

Figure 3.1. Time-dependent effects of c-lact on adrenergic induced *aa-nat* mRNA, protein and enzyme activity. Pinealocytes (1.0×10^5 cells/0.2 ml) were cultured for 24 hours and stimulated with NE (3 μM) for 6 hours with some cells treated with c-lact (10 μM) from 0-3 hours or 3-6 hours of NE treatment. Cells were collected by centrifugation and prepared for RT-PCR, immunoblot or enzymatic activity as described in *Materials and Methods*. Con=control. A) Upper panel: Representative ethidium bromide-stained gels showing *aa-nat* mRNAs. *GAPDH* included to demonstrate loading consistency. Lower panel: Histograms of densitometric measurements of *aa-nat* mRNAs presented as % of maximal OD value. B) Upper panel: Representative immunoblots showing AA-NAT protein. GAPDH included to demonstrate loading consistency. Lower panel: Histograms of densitometric measurements of AA-NAT protein presented as % of maximal OD value. Values represent the mean ± SEM, n=4. C) Histograms of AA-NAT activity. Values represent the mean ± SEM, n=4. *Significantly different from treatment with NE, $P < 0.05$.

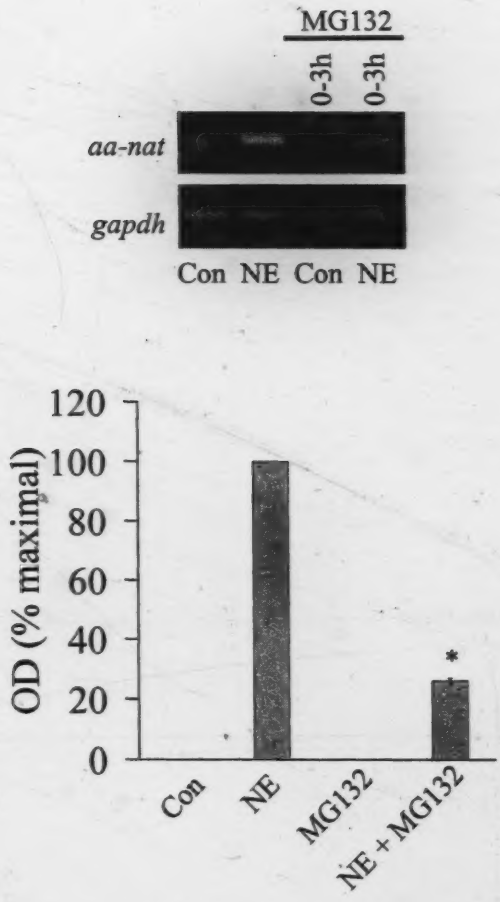

Figure 3.2. Time-dependent effects of MG132 on adrenergic-induced *aa-nat* transcription. Pinealocytes (1.0 x 10^5 cells/0.2 ml) were cultured for 24 h. Cells were stimulated with NE (3 μM) for 6 h with some cells treated with MG132 (3 μM) from 0-3 h of NE treatment. Cells were collected by centrifugation and prepared for RT-PCR as described in *Materials and Methods*. Con=control. Upper panel: Representative ethidium bromide-stained gels showing *aa-nat* mRNAs. *GAPDH* included to demonstrate loading consistency. Lower panel: Histograms of densitometric measurements of *aa-nat* mRNAs presented as % of maximal OD value. Values represent the mean ± SEM, n=3. *Significantly different from treatment with NE, $P < 0.05$.

3.2 Inhibition of AA-NAT is dependent on the dose of MG132 and c-lact

To confirm that the reduction of AA-NAT is dependent on inhibition of the proteasome and that the inhibition corresponds to the dose of the drugs, pinealocytes were simultaneously treated with NE and varying doses of MG132 (3, 1, 0.3, 0.1 μM) or c-lact (10, 3, 1, 0.3 μM). There was a significant inhibition of AA-NAT at the protein level when the cells were treated with 3 μM MG132 (Fig. 3.3a) or 10 μM c-lact (Fig. 3.3b). In addition, as the dose of both drugs was decreased there was less inhibition of AA-NAT. Those cells that were treated with 0.1 μM MG132 or 0.3 μM c-lact showed no inhibition of AA-NAT (Fig. 3.3). Together these results confirm that reduced levels of AA-NAT are a result of proteasome inhibition. Furthermore, it suggests that inhibition of AA-NAT is dependent on the dose of the proteasome inhibitor and that the doses used in this study are appropriate for suppressing *aa-nat* induction.

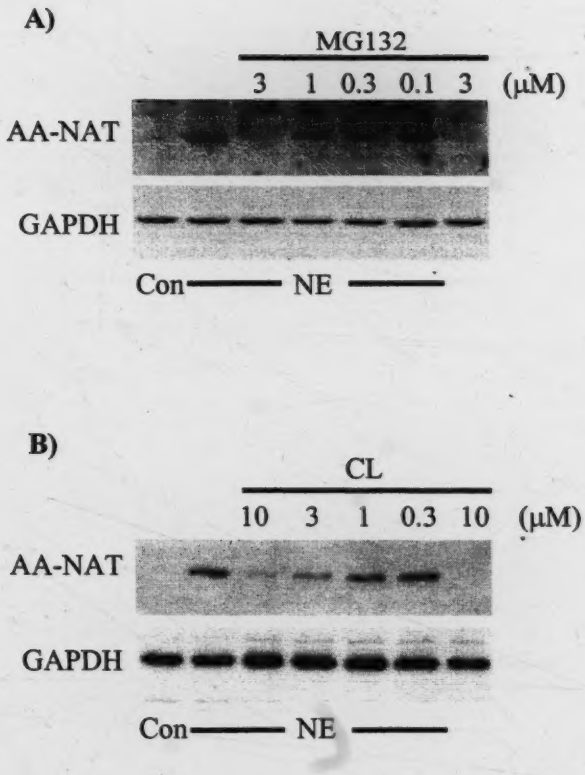

Figure 3.3. Effect of different doses of MG132 and c-lact on AA-NAT protein levels. Pinealocytes (1.0 X 10^5 cells/0.2 ml) were cultured for 24 h and treated with varying doses of MG132 (3, 1, 0.3, 0.1 μM) or c-lact. (10, 3, 1, 0.3 μM). The cells were then stimulated with NE (3 μM) for 3 h and collected for Western blot analysis as described in *Material and Methods*. Con = control. A) Representative immunoblot of cells treated with varying doses of MG132 showing AA-NAT protein, GAPDH included to demonstrate loading consistency. B) Representative immunoblot of cells treated with varying doses of c-lact showing AA-NAT protein, GAPDH included to demonstrate loading consistency.

3.3 Inhibition of *aa-nat* induction by MG132 does not require simultaneous NE stimulation

Since many genes, including repressors, in the pineal gland are induced by NE, it was important to determine whether inhibition of *aa-nat* transcription by a proteasome inhibitor required simultaneous NE stimulation. Pinealocytes were treated with MG132 (3 μM) for 3 hours, followed by washout of MG132 by replacing the cell media, before stimulation with NE (3 μM) for another 3 hours. Levels of *aa-nat* mRNA were significantly reduced when the cells were pre-treated with MG132 (Fig. 3.4).

To characterize the effects of proteasome inhibitors on MT synthesis and to determine if the inhibitory effects were transient or long-lasting, pinealocytes were pre-treated with MG132 (3 μM) or c-lact (10 μM) for 3 hours, followed by washout of inhibitors by replacing the cell media, before stimulation with NE (3 μM) for another 14 hours. Pre-treatment with MG132 or c-lact caused a significant reduction in NE-stimulated AA-NAT activity and MT production (Fig. 3.5). These results suggest simultaneous NE treatment is not required for the inhibitory effect of MG132 on *aa-nat* transcription. Moreover, proteasome inhibitors appear to have a long lasting effect on MT synthesis.

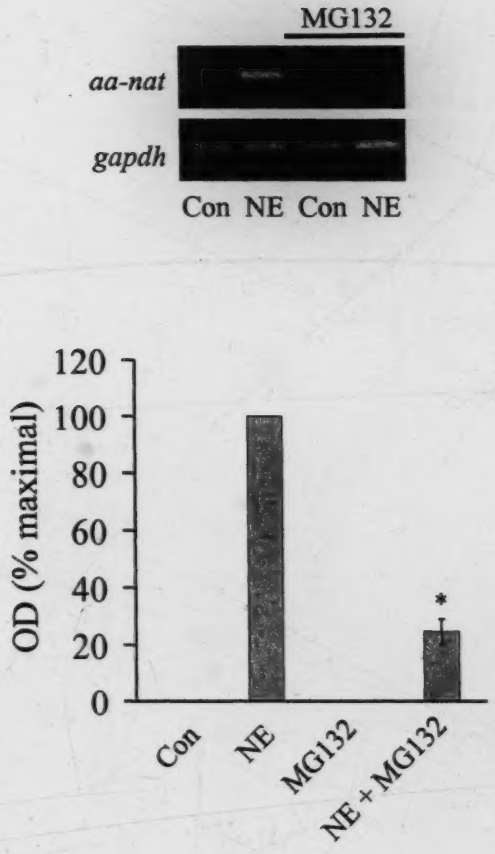

Figure 3.4. Inhibition of *aa-nat* transcription by MG132 does not require simultaneous NE simulation. Pinealocytes (1.0 x 10^5 cells/0.2 ml) were cultured for 24 h and then treated with MG132 (3 μM) for 3 h. Cells were washed with new media and stimulated with NE (3 μM) for another 3 h in the absence of MG132. Cells were collected by centrifugation and prepared for RT-PCR as described in *Materials and Methods*. Con=control. Upper panel: Representative ethidium bromide-stained gels showing *aa-nat* mRNAs. *GAPDH* included to demonstrate loading consistency. Lower panel: Histograms of densitometric measurements of *aa-nat* mRNAs presented as % of maximal OD value. Values represent the mean ± SEM, n=3. *Significantly different from treatment with NE, $P < 0.05$.

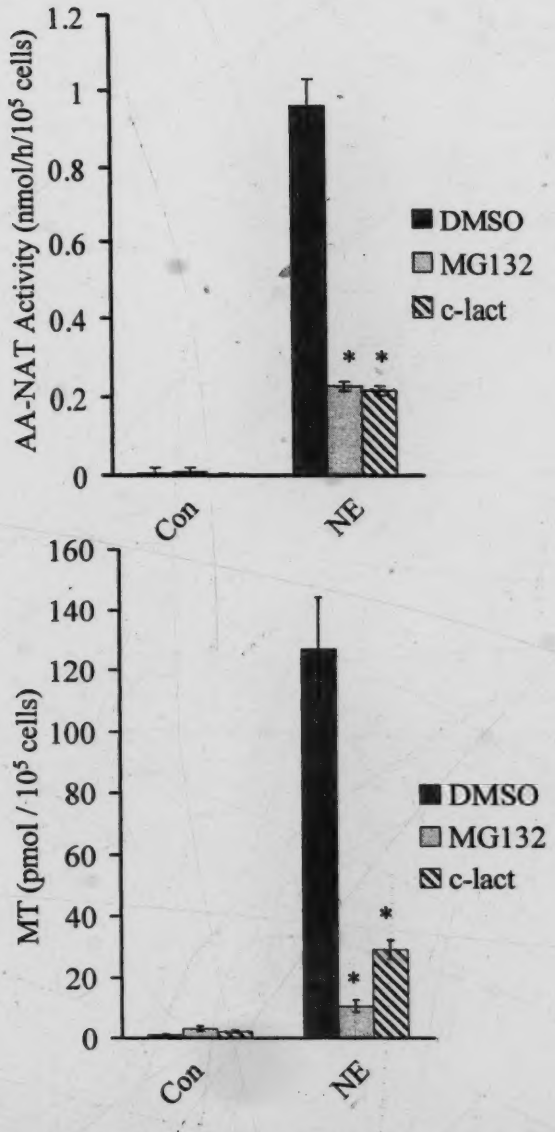

Figure 3.5. Proteasome inhibitors reduce NE-stimulated AA-NAT activity and MT production. Pinealocytes (0.5 X 10^5 cells/0.2 ml) were cultured for 24 h and treated with MG132 (3 μM) or c-lact (10 μM) for 3 h. Cells were washed with new media and stimulated with NE (3 μM) for another 14 h in the absence of proteasome inhibitors. Cells were collected by centrifugation and prepared for AA-NAT enzymatic determination and media were collected for MT production as described in *Materials and Methods*. Con=control. Histograms showing AA-NAT activity (upper panel) and MT production (lower panel). Values represent the mean ± SEM, n=3. *Significant inhibition by the proteasome inhibitor, $P < 0.05$.

3.4 Inhibition of the proteasome after 1, 2 and 3 hours of NE stimulation does not inhibit AA-NAT

Because of the previous result, which demonstrated that blocking proteasomal proteolysis inhibited *aa-nat* transcription only during the early phase of NE stimulation, it was important to determine at what point after NE stimulation the proteasome inhibitors would be unable to inhibit AA-NAT. Since it was previously established that simultaneous NE stimulation is not required for inhibition of *aa-nat* transcription, cells were treated with MG132 (3 μM) 1 hour before NE stimulation as well as 1, 2, and 3 hours after NE stimulation. The cells were stimulated with NE for a total of 6 hours and then collected for Western blot analysis. Those cells that were treated with MG132 1 hour prior to NE stimulation had significantly reduced AA-NAT protein levels (Fig. 3.6). When MG132 was added 1, 2, or 3 hours after NE stimulation there was no reduction in AA-NAT levels compared to those cells treated with NE alone (Fig. 3.6). These results suggest that AA-NAT induction requires proteasomal proteolysis within the first hour of NE stimulation and that inhibition of the proteasome 1 hour after NE treatment has no effect on *aa-nat* transcription.

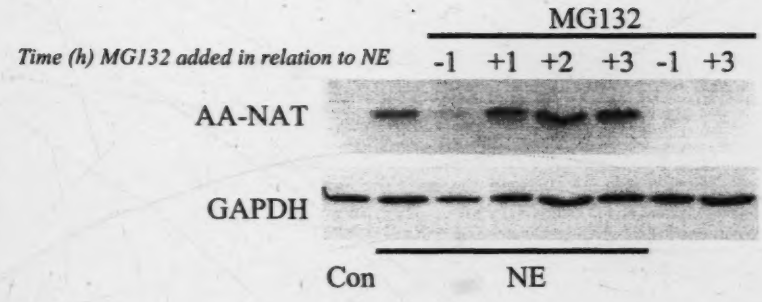

Figure 3.6. Effect of treating cells with MG132 before and after NE stimulation. Pinealocytes (1.0 X 10^5 cells/0.2 ml) were cultured for 24 h. Cells were treated with MG132 (3 μM) 1 h before NE (3 μM) stimulation as well as 1, 2, and 3 h after NE stimulation. The cells were collected after 6 h of NE stimulation and prepared for Western blot analysis as described in *Material and Methods*. Con = control. Representative immunoblot showing AA-NAT protein, GAPDH included to demonstrate loading consistency.

3.5 Effect of proteasomal inhibition on adrenergic-induced transcription is long lasting and selective for *aa-nat*

To determine the duration of inhibition on adrenergic-induced transcription by the proteasome inhibitor, pinealocytes were pre-treated for 3 hours with MG132 (3 μM), followed by replacement with new media that did not contain MG132, and incubation for an additional 14 hours before stimulation with NE for 3 hours. Pre-treatment with MG132 followed by 14 hours of washout remained effective in reducing NE-stimulated increases in *aa-nat* mRNA and protein levels (Fig. 3.7). In contrast, the expression of mitogen activated protein kinase phosphatase-1, *mkp-1*, another adrenergic-regulated gene in the rat pineal gland, was determined and NE was effective in stimulating *mkp-1* transcription in pinealocytes subjected to pre-treatment with MG132 followed by 14 hours of washout (Fig. 3.7). In addition, the inhibition of transcription is selective for *aa-nat*, since the expression of another adrenergic-driven gene, *mkp-1*, is not impaired (Fig. 3.7). Of interest, there was also an increase in basal and adrenergic-stimulated *mkp-1* transcription (Fig. 3.7).

The long lasting effects of the proteasome inhibitors were confirmed using an assay of 20S proteasome activity (Fig. 3.8). These results suggest that the long lasting inhibition of *aa-nat* transcription is due to proteasome inhibition because proteasome activity was suppressed even after 14 hours of washout.

To ensure that the long lasting effects of the proteasome inhibitors on 20S proteasome activity were not due to a reduction in the amount of proteasome in the cell, the levels of α and β-subunits were measured using Western blot analysis. Cells were treated with MG132 (3 μM) for 3 hours, followed by washout for 14 hours before

treatment with NE (3 μM) for 3 hours. There were no changes in the amount of either α or β-subunits after the cells had been exposed to the proteasome inhibitor followed by wash out (Fig. 3.9a). In another experiment, cells were treated with MG132 (3 μM) and NE (3 μM) simultaneously for varying periods to time (7-18 hours) before they were collected for Western blot analysis. Again, there were no changes in the amount of 20S proteasome subunits when the cells were exposed to MG132 for long periods of time (Fig. 3.9b). Together these results suggest that the reduction in 20S proteasome activity from exposure to proteasome inhibitors is a direct result of proteasome inhibition and not a decline in the amount of proteasome present in the cell.

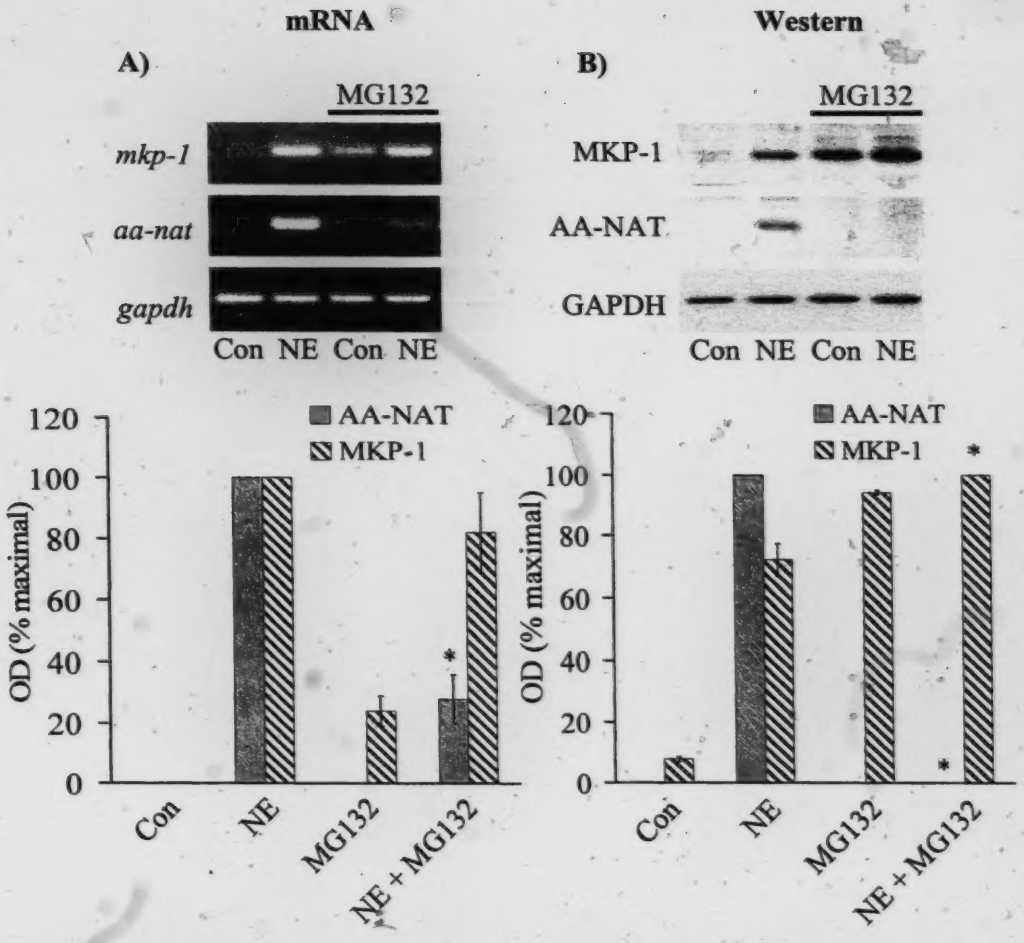

Figure 3.7. Long lasting and selective effect of MG132-mediated inhibition in NE-stimulated *aa-nat* transcription. Pinealocytes (1.0 X 10^5 cells/0.2 ml) were cultured for 24 h and treated with MG132 (3 μM) for 3 h, followed by washout for 14 h before treatment with NE (3 μM) for 3 h. Cells were collected by centrifugation and prepared for RT-PCR or immunoblot as described in *Materials and Methods*. Con=control. A) Upper panel: Representative ethidium bromide-stained gels showing *aa-nat* and *mkp-1* mRNAs, *GAPDH* included to demonstrate loading consistency. Lower panel: Histograms of densitometric measurements of *aa-nat* and *mkp-1* mRNAs presented as % of maximal OD value. Values represent the mean ± SEM, n=4. B) Upper panel: Representative immunoblots showing AA-NAT and MKP-1 proteins, *GAPDH* included to demonstrate loading consistency. Lower panel: Histograms of densitometric measurements of *AA-NAT* and *MKP-1* proteins presented as % of maximal OD value. Values represent the mean ± SEM, n=4. *Significantly different from treatment with NE, $P < 0.05$.

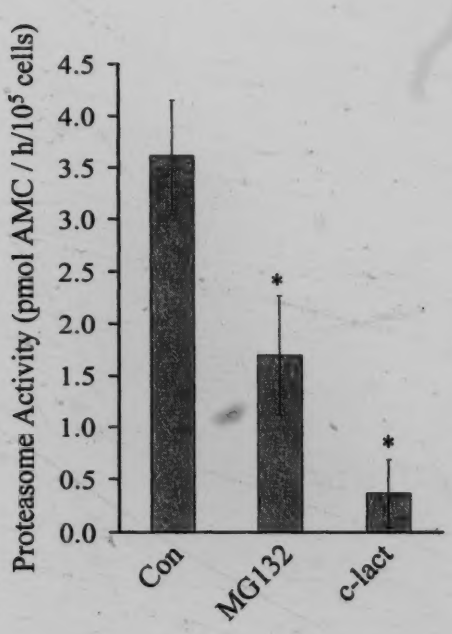

Figure 3.8. Effect of washout on proteasome activity. Pinealocytes (2.0 X 10^5 cells/0.2 ml) were cultured for 24 h and treated with MG132 (3 μM) or c-lact (10 μM) for 3 h. After 14 h of washout, proteasome activity was measured as described in *Materials and Methods*. Con=control. Values represent the mean ± SEM, n=6. *Significantly different from control, $P < 0.05$.

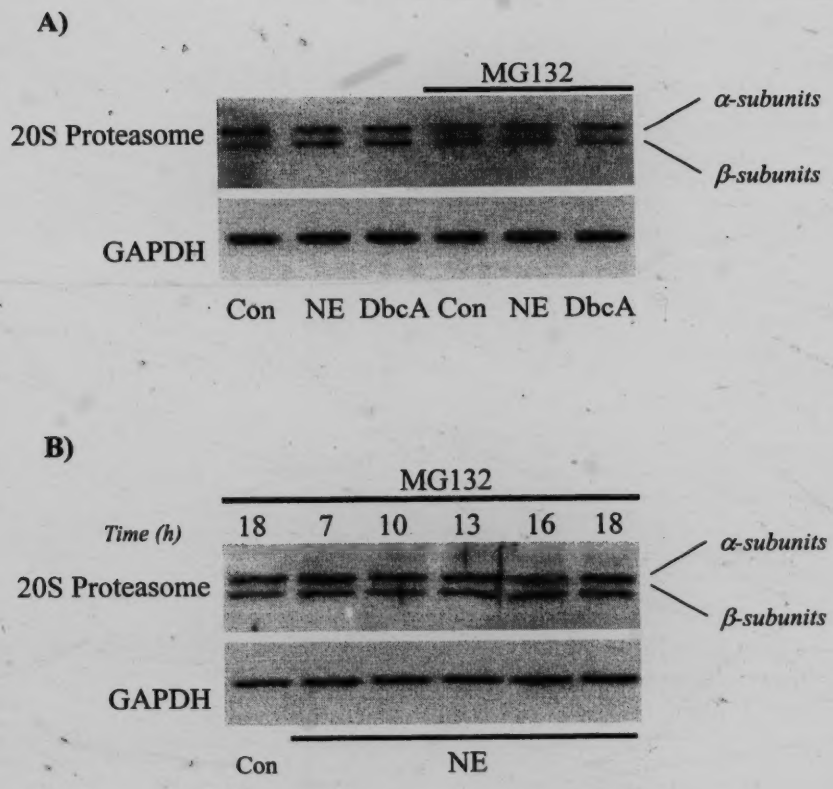

Figure 3.9. Effect of proteasome inhibitors on 20S proteasome subunits. Pinealocytes (1.0 X 10^5 cells/0.2 ml) were cultured for 24 h prior to pharmacological treatments. A) Cells were treated with MG132 (3 μM) then immediately stimulated with either NE (3 μM) or DbcA (1 mM) for 4 hours. The cells were collected for Western blot analysis as described in *Material and Methods*. Con = control. Representative immunoblot showing α- and β-subunits of the 20S proteasome, GAPDH included to demonstrate loading consistency. B) Cells were treated with MG132 for 3 hours then stimulated with NE for varying amounts of time. The cells were collected for Western blot analysis as described in *Material and Methods*. Con = control. Representative immunoblot showing α- and β-subunits of the 20S proteasome, GAPDH included to demonstrate loading consistency. Representative immunoblots, n=3

3.6 Effect of MG132 on the time profile of NE-stimulated *aa-nat* transcription

To determine the time period required for proteasome inhibitors to reduce adrenergic-induced *aa-nat* transcription, cells were treated simultaneously with NE (3 μM) and MG132 (3 μM) for 1, 3 and 5 hours. Treatment with MG132 for 1 hour had no effect on NE-stimulated *aa-nat* mRNA level (Fig. 3.10). However, after 1 hour treatment with NE alone, *aa-nat* mRNA levels continued to increase, whereas treatment with MG132 prevented any further increase in NE-stimulated *aa-nat* mRNA levels (Fig. 3.10). After 5 hours of treatment with MG132 and NE, there was a 65% reduction in *aa-nat* mRNA level compared with cells stimulated with NE alone. This suggests that a lag time of 1 hour is required by the proteasome inhibitor to demonstrate repression of *aa-nat* transcription.

To confirm the requirement of a 1 hour lag time for the inhibition of *aa-nat* transcription, a second time-course study was performed in which pinealocytes were pre-treated with MG132 (3 μM) for 1 hour prior to NE (3 μM) stimulation. Pre-treatment with MG132 reduced NE-stimulated *aa-nat* mRNA levels at 1 and 5 hours by 50% and 75% respectively (Fig. 3.10). This result indicates that a 1 hour pre-treatment is adequate to demonstrate the inhibitory effect of MG132 on *aa-nat* transcription.

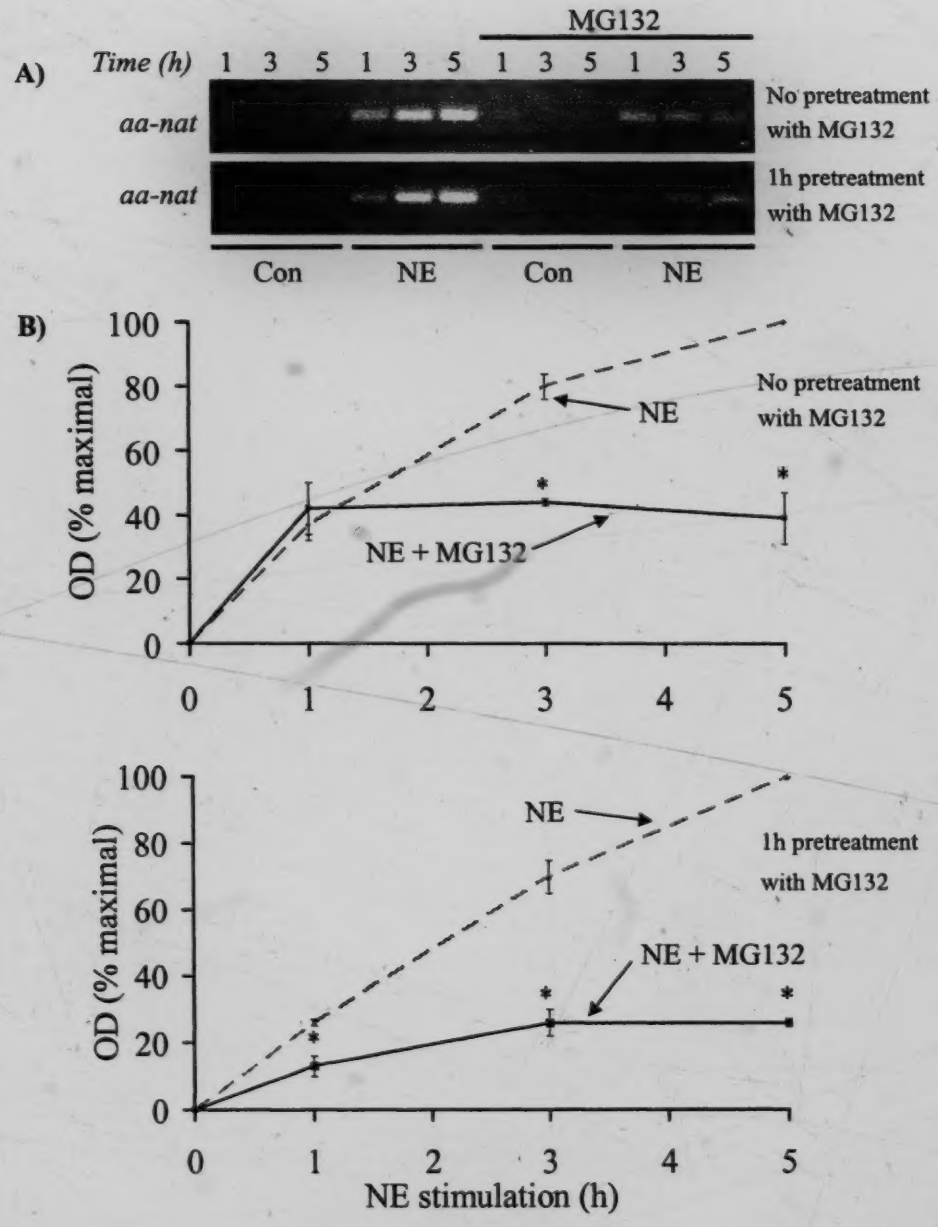

Figure 3.10. Effect of MG132 on the time profile of NE-stimulated *aa-nat* transcription. (see next page for figure legend)

Figure 3.10. MG132 on the time profile of NE-stimulated *aa-nat* transcription. Pinealocytes (1.0 X 10^5 cells/0.2 ml) were cultured for 24 hours and treated with NE (3 μM) for 1 to 5 hours. MG132 (3 μM) was either added simultaneously with NE or 1 hour before treatment with NE. Cells were collected by centrifugation at different time periods and prepared for RT-PCR as described in *Materials and Methods*. Con=control. A) Representative ethidium bromide-stained gels showing *aa-nat* mRNAs. B) Upper panel: Histograms of densitometric measurements of *aa-nat* mRNAs presented as % of maximal OD value from cells treated with MG132 and NE simultaneously. Lower panel: Histograms of densitometric measurements of *aa-nat* mRNAs presented as % of maximal OD value from cells treated with MG132 1 hour prior to the addition of NE. Values represent the mean ± SEM, n=3. *Significant inhibition by MG132, $P < 0.05$.

3.7 Site of action of proteasomal inhibitors on adrenergic-stimulated *aa-nat* transcription

To determine the site of action of proteasomal inhibition on adrenergic induction of *aa-nat* transcription, pinealocytes were pre-treated with MG132 (3 μM) for 3 hours, followed by 14 hours of washout before cells were stimulated for 3 hours with NE (3 μM) or dibutyryl cAMP (1 mM), a cell permeable cAMP analogue. Pre-treatment with MG132 caused a significant reduction in both *aa-nat* mRNA and protein levels in cells that were stimulated with either NE or dibutyryl cAMP (Fig. 3.11). These results indicate that the inhibitory effect of MG132 on *aa-nat* transcription is mediated at a post-cAMP step.

To confirm the result that inhibition of *aa-nat* transcription by proteasome inhibitors is mediated at a post-cAMP step, cells were treated with MG132 (3 μM) or c-lact (10 μM) for 3 hours prior to NE (3μM) stimulation for 15 minutes. Pre-treatment of pinealocytes with proteasome inhibitors for 3 hours did not inhibit the production of cAMP (Fig. 3.12). This result affirms that inhibition of *aa-nat* transcription by proteasome inhibitors occurs at a post-cAMP step and is not caused by a reduction in cAMP production. In addition, it verifies that the concentrations of MG132 and c-lact used in this study did not affect cell viability.

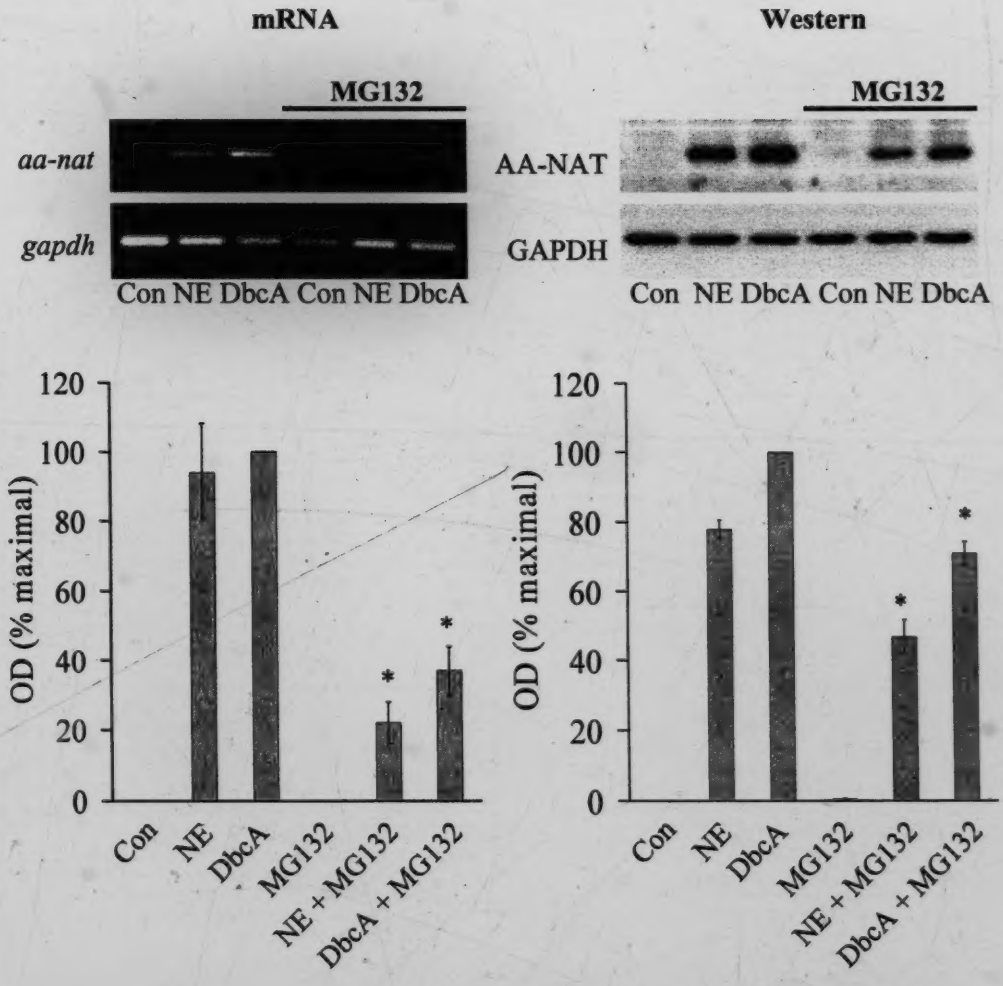

Figure 3.11. Effect of MG 132 on NE- and dibutyryl cAMP-stimulated *aa-nat* transcription and protein levels. Pinealocytes (1.0 x 10^5 cells/0.2 ml) were cultured for 24 h and treated with MG132 (3 μM) for 3 h, followed by washout for 14 h before treatment with NE (3 μM) for 3 h. Cells were collected by centrifugation and prepared for RT-PCR and immunoblot studies as described in *Materials and Methods*. Con=control. Upper panel: Representative ethidium bromide-stained gels and immunoblots showing *aa-nat* mRNAs and protein, *GAPDH* included to demonstrate loading consistency. Lower panel: Histograms of densitometric measurements of *aa-nat* mRNAs and protein presented as % of maximal OD value. Values represent the mean ± SEM, n=4. *Significant inhibition by MG132, $P < 0.05$.

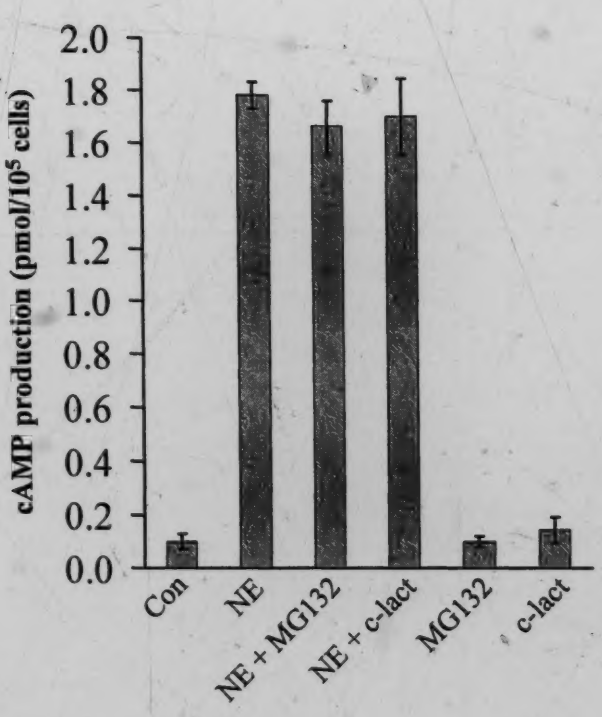

Figure 3.12. Effect of MG132 and c-lact on cAMP production in rat pinealocytes. Pinealocytes (0.2 X 10^5 cells/0.2 ml) were cultured for 24 h and then treated with MG132 (3 μM) or c-lact (10 μM) for 3 hours. After, cells were stimulated with NE (3 μM) for 15 minutes and collected for the cyclic nucleotide assay as described in *Materials and Methods*. Con = control. cAMP production in rat pinealocytes expressed as % maximal of NE stimulated response. Values represent the mean ± SEM, n=8.

3.8 Protein synthesis is involved in the proteasomal inhibitor effects on NE-stimulated *aa-nat* transcription

Considering the primary effect of proteasome inhibitors, a lag time for the demonstration of inhibition of *aa-nat* transcription suggests accumulation of a protein repressor. To examine whether inhibition of *aa-nat* transcription by proteasome inhibitors was due to accumulation of a protein repressor, pinealocytes were treated with a protein synthesis inhibitor, cycloheximide, in the presence or absence of c-lact (10 μM) for 3 hours prior to the addition of NE. Treatment with cycloheximide (30 μg/mL) had an enhancing effect on NE-stimulated *aa-nat* mRNA (Fig. 3.13). The inhibitory effect of c-lact on NE-stimulated *aa-nat* transcription was abolished by co-treatment with cycloheximide (Fig. 3.13). This result suggests that inhibition of *aa-nat* transcription is due to accumulation of a protein repressor and that the inhibitory effect can be abolished by inhibition of protein synthesis.

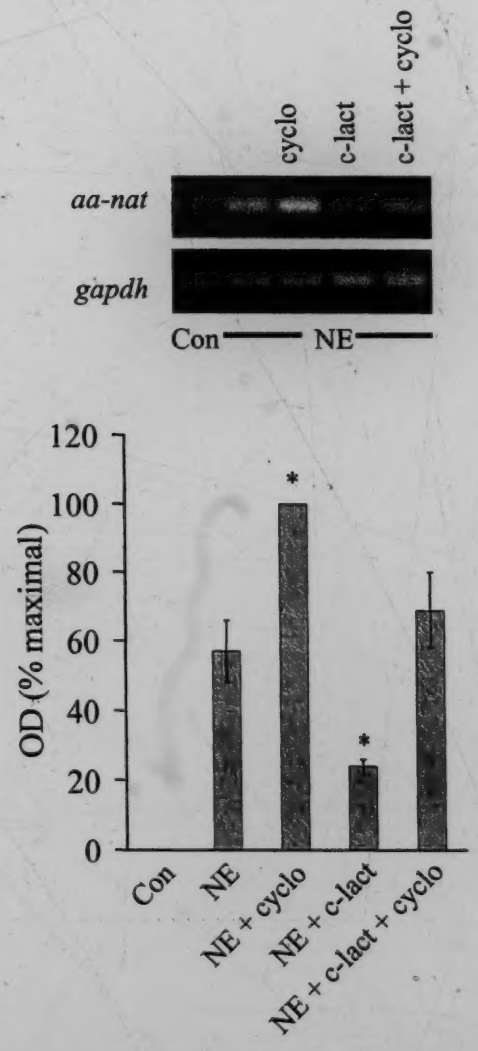

Figure 3.13. Effect of cycloheximide on adrenergic-induced *aa-nat* mRNA. Pinealocytes (1.0 X 10^5 cells/0.2 ml) were cultured for 24 h and treated for 3 h with c-lact (10 μM) and cycloheximide (cyclo, 30 μg/mL) before stimulation by NE (3 μM) for 3 h. Cells were collected by centrifugation and prepared for RT-PCR as described in *Materials and Methods*. Con=control. Upper panel: Representative ethidium bromide-stained gels showing *aa-nat* mRNAs, *GAPDH* included to demonstrate loading consistency. Lower panel: Histograms of densitometric measurements of *aa-nat* mRNAs presented as % of maximal OD value. Values represent the mean ± SEM, n=4. *Significantly different from treatment with NE, $P < 0.05$.

3.9 Proteasome inhibitors have no effect on DREAM in pinealocytes

Previous research has suggested that DREAM is a repressor of *aa-nat* (Link et al., 2004). To determine whether DREAM was the protein that was accumulating in the cell and causing the inhibition of *aa-nat* transcription as a result of blocking proteasomal proteolysis, cells were treated with cycloheximide (30 μg/mL) in the presence of absence of c-lact (10 μM) for 3 hours prior to NE stimulation. Treatment with NE, cycloheximide, or c-lact had no effect on DREAM protein levels (Fig. 3.14a).

In a second experiment, the effects of c-lact on basal and NE-stimulated DREAM protein levels were determined. Cells treated with c-lact (10 μM) and NE (3 μM) simultaneously for 3 hours and then collected for Western blot analysis. Again, c-lact and NE had no effect on DREAM protein levels (Fig. 3.14b). These results suggest that DREAM is not the protein accumulating in pinealocytes and inhibiting *aa-nat* transcription since c-lact had no effect on DREAM. Also, the results indicate that DREAM protein levels may not be regulated by the proteasome.

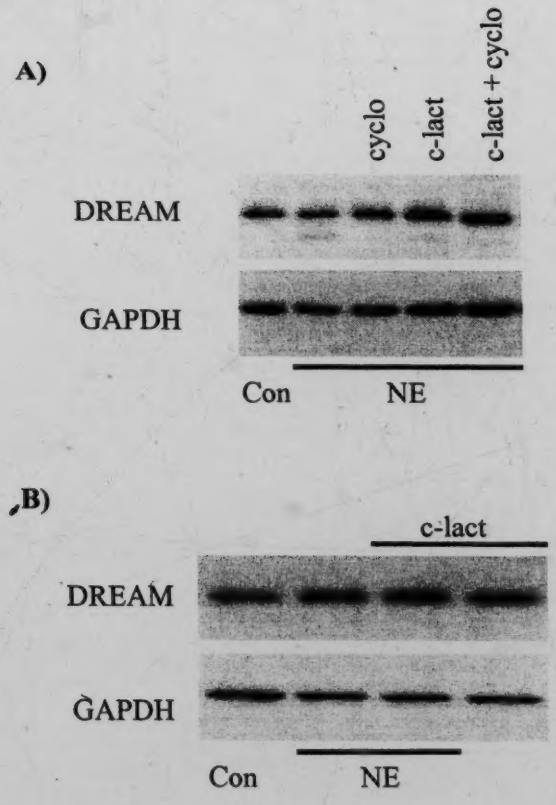

Figure 3.14. Effect of cycloheximide and c-lact on DREAM protein. Pinealocytes (1.0×10^5 cells/0.2 ml) were cultured for 24 h prior to treatment with cycloheximide (cyclo, 30 μg/mL) and c-lact (10 μM). A) Cells were treated for 3 h with c-lact and cyclo before stimulation by NE (3 μM) for 3h. The cells were collected for Western blot analysis as described in *Material and Methods*. Con = control. Representative immunoblot showing DREAM protein, GAPDH included to demonstrate loading consistency. B) Cells were treated simultaneously with NE (3μM) and c-lact (10μM) for 3 h then collected for Western Blot analysis as described in *Materials and Methods*. Con = control. Representative immunoblot showing DREAM protein, GAPDH included to demonstrate loading consistency, n=3.

3.10 Fra-2 expression is inhibited by blocking proteasomal proteolysis

Fra-2 is another transcription factor that may play a role in regulating *aa-nat* transcription (Baler & Klein, 1995; Foletta, 1996; Guillaumond et al., 2000; Nishina, Sato, Suzuki, Sato, & Iba, 1990). To determine if Fra-2 is the protein responsible for inhibiting *aa-nat* transcription as a result of blocking proteasomal proteolysis, cells were stimulated with NE (3 μM) for 6 hours with some cells treated with c-lact (10 μM) from 0-3 hours or 3-6 hours of NE treatment. Fra-2 expression was inhibited when proteasomal proteolysis was blocked for the first 3 hours of NE stimulation (Fig. 3.15a). In addition, when c-lact was added for the last 3 hours of NE treatment then Fra-2 protein levels increased. Also, treatment with c-lact alone did not cause an increase in Fra-2 protein levels. It is interesting to note that there appeared to a slightly larger Fra-2 protein band on the Western blot from cells treated NE alone compared to those cells treated with c-lact (Fig. 3.15a).

To confirm that blocking proteasomal proteolysis inhibits Fra-2 expression, cells were treated with varying doses of c-lact (10, 3, 1, 0.3 μM) for 3 hours then stimulated with NE (3 μM) for 3 hours. Inhibition of Fra-2 was dependent on the dose of c-lact (Fig. 3.15b). Higher doses of c-lact significantly reduce Fra-2 expression whereas lower doses of c-lact did not inhibit Fra-2. Again, a slightly larger Fra-2 protein band was present on the Western blot from cells treated NE alone compared to those cells treated with c-lact (Fig. 3.15b).

Together these results suggest that Fra-2 may not be responsible for the inhibition of *aa-nat* transcription since Fra-2 expression was reduced when c-lact was added for the first 3 hours of NE stimulation. If Fra-2 was the protein involved in suppressing *aa-nat*

transcription then it would be expected that Fra-2 protein levels would increase when cells were exposed to c-lact from 0-3 hours of NE treatment. Fra-2 appears to be regulated by the proteasome in a similar manner to *aa-nat*. Its expression was inhibited by c-lact during the early phase of NE stimulation, yet when c-lact was added for the last 3 hours of NE stimulation Fra-2 protein levels increased. This result also suggests that Fra-2 protein levels are in part regulated by the proteasome. The presence of a slightly larger Fra-2 protein in cells treated with NE alone could be the result of a post-translational modification, which was not present when the cells were treated with c-lact.

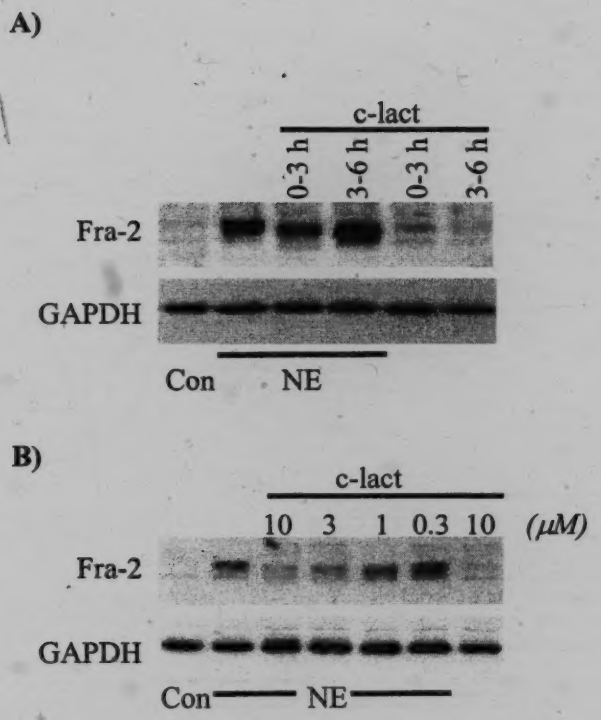

Figure 3.15. Effect of c-lact on Fra-2 protein. Pinealocytes (1.0 X 10^5 cells/0.2 ml) were cultured for 24 h prior to treatment. A) Cells were stimulated with NE (3 μM) for 6 h with some cells treated with c-lact (10 μM) from 0-3 h or 3-6 h of NE treatment. They were collected for Western blot analysis as described in *Materials and Methods*. Con = control. Representative immunoblot showing Fra-2 protein, GAPDH included to demonstrate loading consistency. B) Cells were treated for 1 h with varying doses of c-lact (10, 3, 1, 0.3 μM) then stimulated with NE (3 μM) for 3 h. They were collected for Western blot analysis as described in *Materials and Methods*. Con = control. Representative immunoblot showing Fra-2 protein, GAPDH included to demonstrate loading consistency, n=3.

3.11 ICER expression is inhibited by blocking proteasomal proteolysis

Previous research has suggested that ICER levels are regulated by the proteasome and that ICER inhibits *aa-nat* transcription (Folco & Koren, 1997; Stehle, Foulkes, Molina, Simonneaux, Pevet, & Sassone-Corsi, 1993a). To determine if ICER is the protein responsible for inhibiting *aa-nat* transcription as a result of blocking proteasomal proteolysis, cells were stimulated with NE (3 μM) for 6 hours with some cells treated with c-lact (10 μM) from 0-3 hours or 3-6 hours of NE treatment. It appears that ICER expression was actually inhibited by blocking proteasomal proteolysis within the first 3 hours of NE stimulation (Fig. 3.16a). In addition, like AA-NAT and Fra-2, ICER protein levels increased slightly when c-lact was added from 3-6 hours of NE stimulation.

To confirm that blocking proteasomal proteolysis inhibits ICER expression, cells were treated with varying doses of c-lact (10, 3, 1, 0.3 μM) for 3 hours then stimulated with NE (3 μM) for 3 hours. Again, like AA-NAT and Fra-2, inhibition of ICER may be dependent on the dose of c-lact (Fig. 3.16b). More importantly, treatment of the cells with c-lact alone did not cause an increase in basal ICER protein levels (Fig. 3.16a, Fig. 3.16b)

Together these results suggest that ICER may not be responsible for the inhibition of *aa-nat* transcription when proteasomal proteolysis is blocked since ICER expression was slightly reduced when c-lact was added for the first 3 hours of NE stimulation. If ICER was the protein responsible for inhibiting *aa-nat* transcription then it would be expected that treatment with c-lact alone would result in an increase in ICER protein levels, however this was not observed.

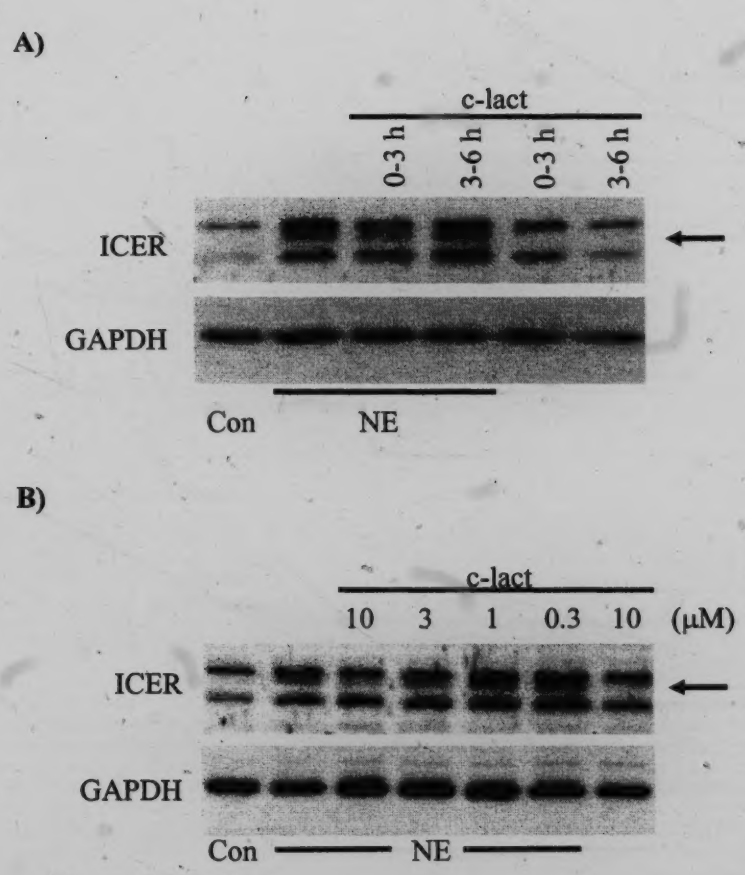

Figure 3.16. Effect of c-lact on ICER protein. Pinealocytes (1.0 X 10^5 cells/0.2 ml) were cultured for 24 h prior to treatment. A) Cells were stimulated with NE (3 μM) for 6 h with some cells treated with c-lact (10 μM) from 0-3 h or 3-6 h of NE treatment. They were collected for Western blot analysis as described in *Materials and Methods*. Con = control. Representative immunoblot showing ICER protein, GAPDH included to demonstrate loading consistency. B) Cells were treated for 1 h with varying doses of c-lact (10, 3, 1, 0.3 μM) then stimulated with NE (3 μM) for 3 h. They were collected for Western blot analysis as described in *Materials and Methods*. Con = control. Representative immunoblot showing ICER protein, GAPDH included to demonstrate loading consistency, n=3.

4. Discussions

4.1 Melatonin and seasonal changes in animal physiology

Changes in seasonal food availability and environmental temperatures are factors that an animal must adapt to in order to maximize its chances of survival and reproduction (Goldman, 1999). The ability to anticipate changes in environmental cues months in advance is a widespread adaptation in nature that helps animals to cope with seasonal climates. Cycles in gonadal activity, moulting, food intake, body weight, and hibernation are annual events that occur in mammals in response to seasonal changes (Nelson & Drazen, 2000; Saarela & Reiter, 1994).

Photoperiod (day length) is the principal factor regulating the timing of these seasonal adaptations in rodents (Arendt, 1998; Nelson, Moffatt, & Goldman, 1994). The amount of light received by the retina is relayed to the pineal gland via a complex neuronal network involving the SCN of the hypothalamus, the PVN, the intermediolateral cell column, and the NE releasing neurons of the SCG (Fig. 1.1) (Klein, 1985). Changes in day length dictate the duration of melatonin release at night (Arendt, Deacon, English, Hampton, & Morgan, 1995). Therefore, in response to long nights, melatonin will be released for a longer period of time than during short nights. It is the change in the duration of melatonin release that is responsible for the seasonal changes in mammalian physiology (Lincoln et al., 2003).

Specialized cells that express high levels of melatonin receptors are able to discriminate between short and long daily exposure to melatonin (Lincoln et al., 2003). As an example, the best characterized group of melatonin sensing cells is the secretory

cells of the pars tuberalis of the pituitary gland, which are responsible for the seasonal regulation of prolactin secretion (Lincoln et al., 2003). Prolactin secretion is higher in the summer and decreased during the winter in response to changes in day light. The seasonal variation in prolactin secretion plays a very important role in regulating the moult cycle, food intake, energy metabolism, gonadal activity, pregnancy and lactation (Hazlerigg, Morgan, & Messager, 2001).

Melatonin plays a very important role in regulating seasonal changes in mammalian physiology. This present study focused on the mechanisms that control the daily rhythm of melatonin production, an essential component of seasonal adaptations. In particular, this study examined the transcriptional regulation of AA-NAT, the rate-limiting enzyme in melatonin production.

4.2 Proteasomal proteolysis and the regulation of AA-NAT

A major factor regulating rhythmic and light-induced changes in AA-NAT activity is the steady state level of AA-NAT protein, which represents a balance of synthesis and degradation (Fig. 1.6). Synthesis of AA-NAT protein is a reflection of adrenergic → cAMP → *aa-nat* mRNA levels, which exhibits a nearly 100-fold nocturnal increase in the rat pineal gland (Roseboom et al., 1996). Based on *in vitro* studies of rat pineal glands, in addition to transcriptional control, proteasomal proteolysis also plays an important role in the circadian and photic regulation of AA-NAT activity (Gastel et al., 1998). In the absence of cAMP, AA-NAT is destroyed by proteasomal proteolysis (Ganguly et al., 2001; Klein et al., 1978) (Fig. 1.6). Using cultured pinealocytes, the present study shows that proteasomal proteolysis, in addition to regulating AA-NAT

protein levels, also plays an important role in the adrenergic induction of *aa-nat* transcription through its effect on transcription repression.

4.3 The balance of repressors and activators in gene regulation

Gene-specific transcriptional repression plays a very important role in gene regulation. In many biological processes, gene expression is a tightly controlled process where genes are expressed only under certain conditions and for specific periods of time. To ensure that a gene is only expressed under the appropriate circumstances, there are many factors such as chromatin remodelling or proteins that interfere with transcription activators that help to repress gene expression (Thiel, Lietz, & Hohl, 2004). Preventing aberrant gene expression is particularly important in cell cycle control. Keeping a gene in the off state can be just as important as turning the gene on.

As an example, the retinoblastoma tumor suppressor gene (pRB) is a negative regulator of cell proliferation that prevents progression from G1 to S phase (Frolov & Dyson, 2004; Thiel, Lietz, & Hohl, 2004). pRB exerts its control over gene expression though interactions with the transcription factor E2F, a protein that regulates several genes responsible for driving the cell cycle from G1 to S phase. In response to appropriate regulatory signals, the inhibitory action on E2F is relieved by pRB phosphorylation and the cell is able to progress through the cell cycle. Knocking out pRB function highlights the importance of suppressing the progression from G1 to S phase. As its name suggests, mutations in the pRB gene result in uncontrolled cellular proliferation. In fact, mutations in pRB have been documented in nearly every type of adult cancer (Sellers & Kaelin, 1997).

Genes regulated by extra-cellular signals are often maintained in a turned off state by repressor proteins until signal transduction alleviates the repression (Courey & Jia, 2001). This is particularly true in the pineal gland where many genes are only expressed in response to NE stimulation (Klein & Weller, 1970) (Fig. 1.3). The pineal gland is a neuroendocrine organ that produces melatonin and it is important that the hormone is only synthesized under the appropriate conditions (Fig. 1.2). Production of melatonin at an inappropriate time could have profound physiological consequences to the organism. Since seasonal changes in an animal's physiology are dependent on slight variations in the duration of melatonin production, it is essential that melatonin synthesis be tightly controlled and directly linked to environmental lighting conditions (Lincoln, Andersson, & Loudon, 2003).

A necessary function of the retinal/SCN/pineal pathway is that the release of NE be intrinsically linked to day length (Fig. 1.1). To ensure that melatonin is only produced in response to NE stimulation, *aa-nat*, the gene controlling melatonin production, must be repressed under basal conditions. It is also necessary to ensure that non-cAMP signals do not activate *aa-nat* transcription since they have the ability to phosphorylate CREB, an essential step in *aa-nat* gene activation. However, CREB phosphorylation alone is not sufficient to activate *aa-nat* transcription (Spessert et al., 2000). The recruitment of CBP appears to be a very important factor for discriminating between cAMP induced and non-cAMP induced signals (B. M. Mayr et al., 2001; Wagner et al., 2000). For example, activation of protein kinase C (PKC) using a specific PKC activator, PMA, resulted in the phosphorylation of CREB, yet it was not sufficient to recruit CBP and activate gene transcription.

Current evidence favours the following model for the regulation of CREB activated transcription (Ernst et al., 2001; B. M. Mayr et al., 2001). This model suggests that an inhibitor, which binds to the KID domain of CREB, may block the association between CREB and CBP. Although the identity of this inhibitor is unknown, it may play a very important role in regulating CREB activated transcription. In particular, this inhibitor may help regulate the transcription of *aa-nat*. It is necessary that the transcription of *aa-nat* be tightly regulated so that it is only expressed at night in response to NE stimulation. This inhibitor, which would be expressed under basal conditions, could play a key role in helping to ensure that *aa-nat* is expressed only at night by NE stimulation.

4.4 Time dependent effects of proteasome inhibitors on *aa-nat* transcription

The time-dependent effect of treatment with proteasome inhibitors on adrenergic-induced transcription of *aa-nat* is of interest. Inhibition of the proteasome prior to or concurrent with NE stimulation causes a significant reduction in *aa-nat* mRNA and protein levels (Fig. 3.1). In contrast, when the proteasome is inhibited after stimulation with NE, it enhances the adrenergic-stimulated AA-NAT protein levels as reported in a previous study without having an effect on *aa-nat* mRNA (Gastel et al., 1998) (Fig. 3.1). Moreover, similar time-dependent effects of the proteasome are obtained with two structurally unrelated proteasome inhibitors, MG132 and c-lact (Fig. 3.2). Data from this present study suggest that proteasome activity is only required for the first hour of *aa-nat* induction, since the addition of proteasome inhibitors after 1 hour of NE stimulation did not result in a reduction of AA-NAT protein (Fig. 3.6). Taken together, my results

indicate that these time-dependent effects of proteasome inhibitors are mediated through inhibition of transcription and not degradation of *aa-nat* mRNA. Whether proteasomes exert an enhancing or inhibitory effect on adrenergic-regulated AA-NAT protein levels is critically dependent on the time of exposure to proteasome inhibitors. Once transcription has been initiated, it appears that the proteasome has little or no effect on transcription. However, if proteasomal proteolysis is inhibited prior to the initiation of *aa-nat* transcription then *aa-nat* transcription will be significantly impaired.

4.5 *Aa-nat* repression under basal conditions

In addition, the inhibitory effect of proteasomal inhibition on *aa-nat* transcription does not require continuous stimulation with NE (Fig. 3.4, Fig. 3.5). This is a surprising result since many genes in the pineal gland are induced by NE stimulation (Klein, 1985). As an example, ICER and Fra-2, two suspected repressors of *aa-nat* transcription, are significantly up-regulated in response to NE stimulation (Baler & Klein, 1995; Folco & Koren, 1997; Stehle, Foulkes, Molina, Simonneaux, Pevet, & Sassone-Corsi, 1993a). The result suggests that a non-cAMP regulated factor is inhibiting *aa-nat* transcription when proteasomes are inhibited. Also, this result highlights the importance of repressing *aa-nat* transcription during the basal state. It is important to prevent *aa-nat* transcription during the day and to ensure that *aa-nat* is expressed only in response to NE stimulated. Therefore, a tight balance between activators and repressors is likely necessary to ensure that *aa-nat* is expressed by the appropriate signal and at the correct time. This hypothesis has been supported by other findings. Previous studies found that CREB phosphorylation alone is not sufficient to induce *aa-nat* expression, suggesting the involvement of a

repressor (Spessert et al., 2000). In addition, non-cAMP signals are unable to induce *aa-nat* transcription, yet they are able to phosphorylate CREB (Sun, Enslen, Myung, & Maurer, 1994).

During the day, under basal conditions, it is plausible that there are factors being expressed, which inhibit *aa-nat* transcription. However, in response to NE stimulation, the activating factors counteract and overcome the threshold set by the repressive factors and the gene is transcribed. The proteasome may play a role in this process by regulating the level of a repressive factor. In response to NE, it is plausible to speculate that the repressive factor may be post-translationally modified which would allow it to be recognized by a ubiquitin ligase. Interaction with a ubiquitin ligase triggers a substrate for degradation by the proteasome. As an example, mitotic cyclins, involved in regulating the cell cycle, are targeted for degradation by the proteasome after being phosphorylated. Phosphorylation allows them to be recognized by a specific ubiquitin ligase, which catalyzes the ubiquitination of the cyclins (Ciechanover, Orian, & Schwartz, 2000). As a result of NE stimulation, a repressor of *aa-nat* may be selectively degraded by the proteasome, which would facilitate *aa-nat* expression. Another possibility is that a balance between constant synthesis and degradation of a repressor may result in a steady state level of the repressor protein in the cell and create a threshold for *aa-nat* activation. Again, it would require a sufficient stimulus from NE to overcome this threshold and initiate transcription.

4.6 Long-lasting inhibitory effect on *aa-nat* transcription

Treatment with the proteasome inhibitor MG132 also has a long lasting effect on adrenergic-stimulated *aa-nat* induction that remains after 14 hours of washout (Fig. 3.7). The sustained effect was confirmed using a 20S proteasome activity assay in rat pinealocytes which showed that inhibition of proteasome activity is irreversible *in vivo* (Fig. 3.8). This was an unexpected result since MG132 is a proteasome substrate analog that binds reversibly to the catalytic core *in vitro*. In addition, a study in *Saccharomyces* demonstrated that the inhibitory effect of MG132 is reversible *in vivo* (Lee & Goldberg, 1996b). However, it is important to note that although the proteasome is a highly conserved complex, there may be subtle structural differences between the yeast and rat proteasomes that could explain the irreversibility of MG132 inhibition. To exclude the possibility that the sustained reduction in proteasome activity was due to a down-regulation of the 20S proteasome subunits, the amount of α and β-subunit were measured by Western blotting. There were no reductions in the amount of either subunit suggesting that the long-lasting effect on AA-NAT induction was due to irreversible inhibition of the proteasomes activity by MG132 (Fig. 3.9).

My results suggest proteasome turnover in pinealocytes is likely a very slow process since *de novo* synthesis of functional proteasomes in the cell should lead to reversal of inhibition of adrenergic-induced *aa-nat* transcription. This contradicts a previous study that suggested inhibition of proteasomes with MG132 and c-lact results in *de novo* synthesis of proteasomes in mammalian cells (Meiners et al., 2003). Surprisingly, they observed *de novo* synthesis of both α and β subunit mRNAs and proteins after only 6 h of exposure to proteasome inhibitors. It is worthwhile to note that

they used cancer cell lines as opposed to primary cultured cells, which could explain the discrepancy between our results. In addition, it is possible that *de novo* proteasome synthesis is very slow is some cell types, including the pineal gland. This is a plausible explanation since protein turnover likely varies widely among different cell types. Nonetheless, the sustained inhibition of *aa-nat* confirms the importance of proteasomal proteolysis in *aa-nat* gene expression in the rat pineal gland.

4.7 Site of action of proteasomes on *aa-nat* transcription

My results also provide evidence on the site of action of proteasomes on adrenergic induced *aa-nat* transcription. Inhibition of the proteasomes by MG132 causes a similar inhibition in NE- or dibutyryl cAMP-stimulated *aa-nat* transcription, indicating that the proteasome inhibitors are likely acting at a post-cAMP step distal to the adrenergic receptor (Fig. 3.11). This result was further supported by cAMP measurements, which demonstrated that neither MG132 nor c-lact caused a reduction in cAMP production (Fig. 3.12). Since cAMP production remained intact in the presence of proteasome inhibitors, it confirms that the effects of blocking proteasomal proteolysis on *aa-nat* transcription are mediated at a post-cAMP step. In all likelihood, unless PKA activity is being affected, the effects of MG132 and c-lact on *aa-nat* transcription are occurring in the nucleus, at the transcriptional level.

4.8 Why is inhibition by proteasomes selective? A comparison of *aa-nat* and *mkp-1* promoters

Of interest, this long lasting effect of proteasomes is selective for *aa-nat* and is not shared by another adrenergic-regulated gene, mitogen activated protein kinase phosphatase–1 (*mkp-1*). MKP-1 is a dual specificity phosphatase that inactivates mitogen activated protein kinases. Previous studies have shown that *mkp-1* is highly expressed in the rat pineal gland at night and displays a diurnal rhythm (Price et al., 2004). In particular, stimulation of pinealocytes with dibutyryl cAMP, a cell permeable cAMP analog, resulted in a significant increase in *mkp-1* expression (Price, Chik, & Ho, 2004). This study suggests that *mkp-1* is regulated via the cAMP pathway in a similar manner to *aa-nat*. However, the current results suggest that there are some differences in the adrenergic regulation of *aa-nat* and *mkp-1* transcription or alternatively a difference in their stability and transcription efficiency.

A comparison of the response by *mkp-1* and *aa-nat* to inhibition of proteasomal proteolysis provides a method to examine the mechanism behind inhibition of *aa-nat* transcription. By comparing the promoters of the two genes, it is possible to shed light on how proteasomal proteolysis affects the cAMP-driven transcription of *aa-nat*. Although both genes are induced by cAMP and contain CRE sites, there are some fundamental differences between their promoters.

Unlike *mkp-1*, the *aa-nat* promoter contains a regulatory site referred to as a CCAAT box (Baler et al., 1997; Baler, Covington, & Klein, 1999). This type of regulatory element has been found in many promoters and appears to control gene expression through interactions with CCAAT enhancer binding proteins (C/EBPs)

(Chodosh, Baldwin, Carthew, & Sharp, 1988). In the *aa-nat* promoter, the CCAAT element seems to exert a positive effect on transcription since site directed mutagenesis abolished binding activity and eliminated reporter gene activity (Baler et al., 1997). There are also reports in the literature that cooperation occurs between CCAAT and CRE sites. In the fibronectin gene promoter, occupancy of the CCAAT site in facilitated by the CRE (Muro, Bernath, & Kornblihtt, 1992). These results indicate that both the CCAAT and CRE sites facilitate the activation of some cAMP induced genes.

One exception is *mkp-1*. Although m*kp-1* is regulated via the cAMP → PKA → CREB pathway and contains two CRE sites within its promoter, it lacks a CCAAT site. Instead, the *mkp-1* promoter contains two GC-rich elements, which are though to work synergistically with CRE sites (Ryser, Massiha, Piuz, & Schlegel, 2004). When the GC-rich elements were mutated by site directed mutagenesis, the promoter was unable to respond to stimulation. This suggests that these GC-rich elements are essential for cAMP regulated *mkp-1* expression.

Since *mkp-1* is expressed at low levels in the pineal under basal conditions, its transcription is likely not highly repressed. *Aa-nat* expression on the other hand is tightly regulated. The cooperation between the CRE sites and CCAAT elements may play a role in repressing *aa-nat* transcription under basal conditions. There is a large family of transcription factors, which bind to CCAAT elements called CCAAT/enhancer-binding proteins (C/EBPs) (Landschulz, Johnson, & McKnight, 1988; Landschulz, Johnson, & McKnight, 1989). Like CREB, they belong to a family of basic leucine zipper (bZIP) DNA binding proteins that are involved in regulating a wide variety of processes such as inflammatory signals and cell differentiation (Lekstrom-Himes & Xanthopoulos, 1998).

C/EBPs likely play a role in regulating adrenergic induced *aa-nat* transcription since mutation of the CCAAT element in the *aa-nat* promoter results in a 70% reduction in transcription (Baler et al., 1997). There are two transcriptional activators in this family called C/EBP-α and C/EBP-β which are expressed in the rat pineal gland (Klein, DC, personal communication, March 24, 2005). They bind as heterodimers to DNA and activate transcription likely through interactions with CREB (Kinoshita, Akira, & Kishimoto, 1992; Williams, Cantwell, & Johnson, 1991). Although their physiological role in the pineal has not been examined, it is logical to assume that C/EBP-α and β are involved in regulating *aa-nat* transcription.

Another difference between *aa-nat* and *mkp-1* is that *mkp-1* is expressed under basal conditions in the pineal. Blocking proteasomal proteolysis resulted in a large accumulation of MKP-1 protein even in cells that were not stimulated. However, under the same conditions *aa-nat* was not expressed. Previous studies have suggested that GC-rich elements are important for basal *mkp-1* transcription (Ryser et al., 2004). The presence of these elements in the *mkp-1* promoter and not the *aa-nat* promoter could be an important factor in determining the different expression pattern of these two genes.

4.9 Involvement of a protein repressor in proteasome mediated *aa-nat* inhibition

The effect of proteasomes on *aa-nat* transcription appears to be mediated by the accumulation of a protein repressor. This is suggested by the observation that a lag time of 1 h is required by the proteasome inhibitor to cause inhibition of *aa-nat* transcription (Fig. 3.10). Although some data has suggested that proteasome inhibitors can take as long as 30 min to inhibit the proteasome, this does not explain the 1 h lag time required to

repress *aa-nat* transcription (Lee & Goldberg, 1996). It may be that for the first 30 minutes of exposure to the proteasome inhibitor the proteasome is still functional, but after that period a protein repressor may be accumulating.

To confirm that a protein repressor was building up in the cells, the effect of blocking protein synthesis on *aa-nat* expression was examined. Inhibition of *aa-nat* transcription by proteasome inhibitors can be abolished by treatment with cycloheximide, a protein synthesis inhibitor (Fig. 3.13). This result suggests the build-up of a protein repressor that requires continuous synthesis. It suggests that under basal conditions, a protein repressor of *aa-nat* is being constitutively synthesized and degraded by the proteasome. The balance between constant synthesis and degradation may result in a steady state level of repressor in the cell and create a threshold for activation. By blocking protein translation, it prevents the synthesis of the repressor and therefore prevents the inhibition of *aa-nat* transcription. Inhibiting proteasomal proteolysis likely prevents the degradation of the repressor and allows it to accumulate in the cell. This may result in a higher threshold for activation and lead to inhibition of *aa-nat* transcription.

4.10 Role of DREAM in proteasome mediated *aa-nat* repression

Previous research has suggested that DREAM may be responsible for repressing *aa-nat* transcription therefore the effect of proteasome inhibitors on DREAM protein levels were examined (Link et al., 2004). DREAM is a Ca^{2+} regulated transcriptional repressor that binds to the downstream regulator element (DRE) site (GAGTCAAGG) in the *aa-nat* promoter and possibly represses transcription (Osawa et al., 2001; Osawa et al., 2005). DREAM has been shown to block the recruitment of CBP by CREB, which

could be a possible mechanism for repressing *aa-nat* transcription (Ledo, Carrion, Link, Mellstrom, & Naranjo, 2000; Ledo, Kremer, Mellstrom, & Naranjo, 2002).

My results indicate that DREAM protein levels are not regulated by the proteasome since treatment of pinealocytes with c-lact had no effect on DREAM (Fig. 3.14). In addition, blocking protein synthesis did not cause a reduction in DREAM which suggests that the protein is very stable and that continuous protein synthesis is not required to maintain steady state levels of the protein. Based on these results, it is logical to conclude that DREAM is not regulated by the proteasome and that it is not the protein repressor responsible for inhibiting *aa-nat* transcription after proteasomal proteolysis has been blocked.

4.11 Role of Fra-2 in proteasome mediated *aa-nat* repression

The regulation of Fra-2 by the proteasome was also examined since previous research had suggested that the transcription factor might be involved in repressing *aa-nat* transcription (Baler & Klein, 1995). Evidence for the control of *aa-nat* by Fra-2 came from promoter studies, which determined that the *aa-nat* promoter contains a putative AP-1 site, the binding element for Fra-2. This suggested that Fra-2 may bind to the AP-1 site and repress *aa-nat* transcription (Baler & Klein, 1995). To determine if Fra-2 played a role in *aa-nat* transcription, I examined whether a decrease in *aa-nat* expression resulting from inhibiting the proteasome correlated with an increase in Fra-2 protein. Interestingly, levels of Fra-2 protein decreased in the presence of c-lact (Fig. 3.15). This suggests that Fra-2 is regulated in a similar manner to the *aa-nat* gene and that it likely does not inhibit *aa-nat* transcription. It is possible that the *fra-2* promoter contains a

CCAAT site and that the presence of this element accounts for its regulation by the proteasome. Further research will have to be done on the *fra-2* promoter to determine if a CCAAT site is present and what functional role this element plays in the rat pineal gland.

It is worthwhile to note that there was a shift in the size of the Fra-2 protein band on Western blots when cells were exposed to proteasome inhibitors. Previous evidence had suggested that Fra-2 undergoes post-translational modification such as phosphorylation (Murakami et al., 1997). A shift in band size could be explained by a change in its phosphorylation state, which in turn could affect its behaviour as a transcription factor, since research has demonstrated that Fra-2 can act as an activator of its own transcription when it is phosphorylated (Murakami et al., 1997). This result provides some evidence that Fra-2 is regulated by the proteasome, yet the mechanism is unclear. It will be interesting to determine how the proteasome affects the post-translational modifications of Fra-2 and what physiological role this plays in pinealocytes.

4.12 Role of ICER in proteasome mediated *aa-nat* repression

Another possible candidate for the protein repressor is ICER. Previous studies suggest that ICER may be involved in repressing *aa-nat* transcription at the end of the night because ICER is expressed diurnally and binds to CRE sites (Blendy, Kaestner, Weinbauer, Nieschlag, & Schutz, 1996; Foulkes et al., 1996; Kell et al., 2004; Maronde et al., 1999). Steady state levels of ICER are believed to be regulated both by protein synthesis and degradation by the proteasome (Zheng et al., 2003). Because the amount of ICER protein present in the nucleus can affect the rate of transcription of cAMP-

regulated genes, the proteasome may play an important role in the control of adrenergic-regulated genes through its effect on ICER (Stehle, Foulkes, Molina, Simonneaux, Pevet, & Sassone-Corsi, 1993a). However, my results indicate that the inhibitory effect of proteasomal inhibition on *aa-nat* transcription does not require continuous stimulation with NE (Fig. 3.4). In addition, inhibition of the proteasome appears to cause a slight decrease in ICER protein levels in the early phase of NE stimulation. This suggests that the transcription of *fra-2*, *icer* and *aa-nat* may be regulated by a similar mechanism and that ICER may not be the repressor protein. It may be that ICER plays more of a role in repressing *aa-nat* expression at the end of the night as opposed to the early phase of the night. Also, ICER may only be regulated by the proteasome at a particular point in the evening, which may explain why inhibition of the proteasome during the early phase of NE stimulation does not cause an increase in ICER levels.

4.13 Potential role of C/EBP-γ in proteasome mediated *aa-nat* repression

A likely candidate for a repressor protein which may regulate *aa-nat* expression under basal conditions and during the early phase of the night is a truncated version of C/EBP-α and β called C/EBP-γ (Cooper, Henderson, Artandi, Avitahl, & Calame, 1995). C/EBP-γ does not contain the amino terminal activator domain found in its non-truncated family members and hence acts as a dominant repressor of C/EBP mediated transcription. Studies have shown that C/EBP-γ forms a heterodimer with both C/EBP-α and β and creates a transcription complex which is able to bind to CCAAT sites, but is unable to initiate transcription (Parkin, Baer, Copeland, Schwartz, & Johnson, 2002).

A likely model for the physiological function of C/EBP-γ in pinealocytes is to set the threshold for activation of C/EBP-α and β regulated genes. In all cell types examined, C/EBP-γ is constitutively expressed. Therefore, under non-stimulated conditions, C/EBP-γ would act as a buffer of C/EBP activation to ensure that *aa-nat* remains turned off during the day (Parkin et al., 2002). However, under stimulated conditions, both C/EBP-α and β, which display a diurnal expression rhythm, would accumulate in the cell and overcome the threshold, set by C/EBP-γ.

Recently, it has been shown that the level of C/EBP-γ is regulated by the ubiquitin-proteasome system (Hattori, Ohoka, Inoue, Hayashi, & Onozaki, 2003). Since C/EBP-γ is constitutively expressed in the cell, a constant balance between protein synthesis and degradation would be required to maintain the activation threshold. This balance between synthesis and degradation is supported by the finding that blocking proteasomal proteolysis with either MG132 or c-lact caused a significant increase in C/EBP-γ protein (Hattori, Ohoka, Inoue, Hayashi, & Onozaki, 2003). In addition, blocking protein synthesis with cycloheximide resulted in reduced levels of C/EBP-γ, suggesting that it is constantly being translated (Hattori et al., 2003).

It was also demonstrated that C/EBP-γ is multi-ubiquitinated *in vivo* (Hattori et al., 2003). In the pineal, this multi-ubiquitination may contribute to its rapid degradation in response to NE stimulation and prevent the build-up of excess inhibitory signal. It is important to note that the ubiquitin ligase responsible for targeting C/EBP-γ for degradation only recognizes the monomeric form. When C/EBP-γ is bound to either C/EBP-α or β, it is protected from degradation. This may be a mechanism to eliminate

any transcriptionally non-functioning C/EBP-γ. It has also been argued that C/EBP-γ may be phosphorylated to facilitate its binding to other C/EBP family members (Parkin et al., 2002). If this were true then rapid dephosphorylation of C/EBP-γ may be a signal to target the protein for proteasomal degradation.

Several key findings suggest a role for C/EBP-γ in adrenergic induced *aa-nat* transcription. First, C/EBP-γ is expressed in rat pinealocytes and its levels in other cells are regulated by the proteasome (Klein, DC, personal communication, March 24, 2005; Hattori, Ohoka, Inoue, Hayashi, & Onozaki, 2003). This body of research has demonstrated that a protein regulated by the proteasome represses *aa-nat* transcription. Second, C/EBP-γ is not regulated by NE and is constitutively expressed (Klein, DC, personal communication, March 24, 2005). This is in line with the results presented in this thesis, which demonstrated that *aa-nat* repression may not require simultaneous NE stimulation and is likely mediated by a protein that is not under the regulation of NE. Third, inhibition of proteasomal proteolysis using MG132 of c-lact caused an accumulation of C/EBP-γ by inhibiting its degradation (Hattori et al., 2003). My data demonstrated that in rat pinealocytes, inhibiting protein synthesis with cycloheximide abolished the effects of MG132 and c-lact on *aa-nat* transcription. This suggests that preventing the synthesis of the protein repressor counteracts the effects of blocking proteasomal proteolysis and prevents the accumulation of the repressor.

5. Summary

Inducible genes are under the control of both activating and repressive signals (Foulkes, Whitmore, & Sassone-Corsi, 1997; Molina et al., 1993). A tight balance between repression and activation of transcription likely regulates the amplitude and duration of *aa-nat* gene expression. Based on previous studies, it is clear that proteasome activity can dramatically influence gene expression. With these findings in mind, it was decided to examine whether the proteasome regulated the transcription of *aa-nat*. Using the specific proteasome inhibitors, MG132 and c-lact, this study examined the consequences of blocking proteasomal proteolysis on adrenergic induced *aa-nat* transcription, AA-NAT protein levels, AA-NAT enzymatic activity and melatonin production.

In the mammalian pineal gland, adrenergic regulation of AA-NAT, is in part mediated by rapid reversible control of proteasomal proteolysis. Blocking proteasomal degradation after *aa-nat* transcription has been initiated results in an increase in AA-NAT protein (Fig. 4.1). However, inhibition of the proteasome prior to the initiation of *aa-nat* transcription causes a significant reduction in *aa-nat* mRNA and protein levels (Fig. 4.2).

This body of work provides the first evidence that a repressor protein, regulated by the proteasome, is directly involved in controlling the transcription of *aa-nat* in the rat pineal gland. This thesis demonstrates that: 1) proteasomal proteolysis is required for *aa-nat* transcription during the early phase of NE stimulation; 2) inhibition of *aa-nat* transcription is not dependent on simultaneous NE stimulation; 3) the effects of proteasome inhibitors on adrenergic induced transcription are long-lasting; 4) inhibition of the proteasome with MG132 and c-lact selectively inhibits *aa-nat* transcription and not

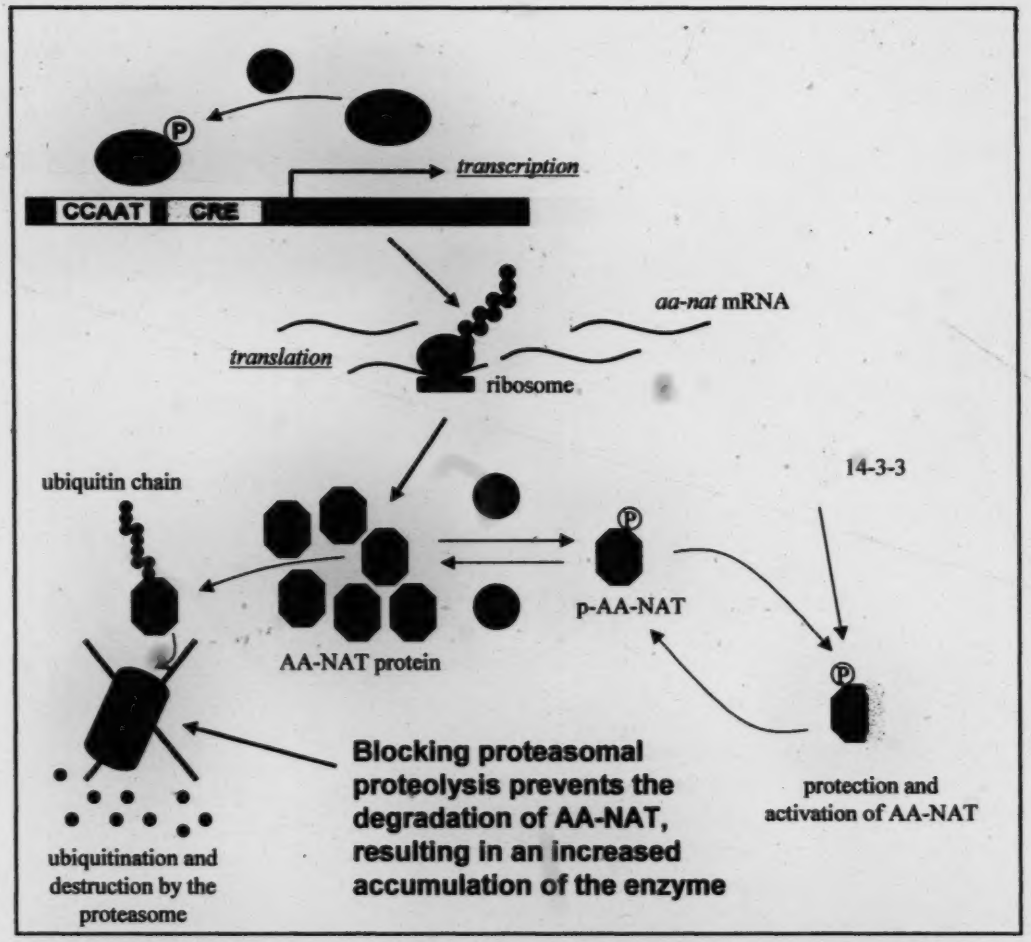

Figure 4.1 Inhibition of proteasomal proteolysis after *aa-nat* transcription initiation. Synthesis of AA-NAT protein is a reflection of cAMP → pCREB → *aa-nat* mRNA levels, which exhibits a nearly 100-fold nocturnal increase in the rat pineal gland. Proteasomal proteolysis also plays an important role in the circadian and photic regulation of AA-NAT activity. Blocking proteasomal proteolysis after *aa-nat* transcription has been initiated results in an increased accumulation of AA-NAT protein since its degradation is prevented.

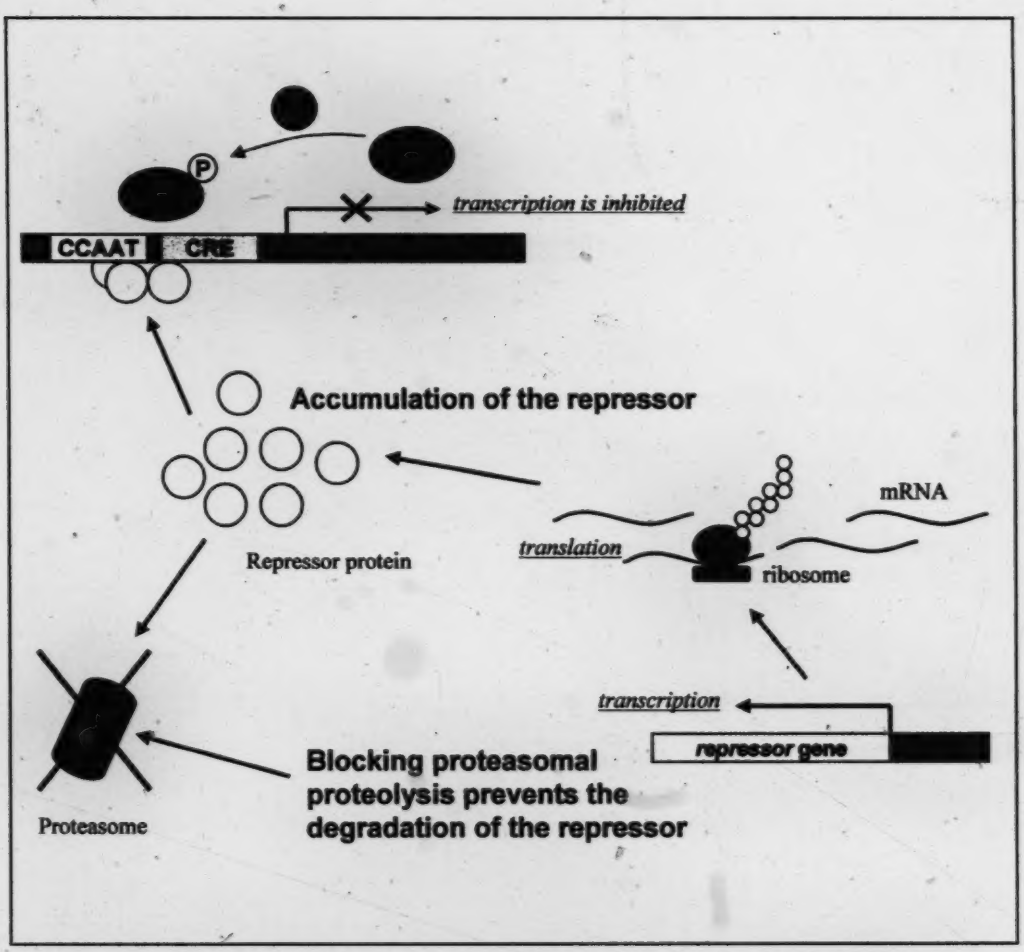

Figure 4.2 Inhibition of proteasomal proteolysis prior to *aa-nat* transcription initiation. Blocking proteasomal proteolysis may prevent the degradation of a repressor of *aa-nat* and lead to its accumulation in the cell. Increased levels of the repressor would lead to the inhibition of *aa-nat* transcription. Based on data in this present study, it is unlikely that DREAM, Fra-2, or ICER is preventing *aa-nat* transcription through a proteasome dependent mechanism. One likely candidate for the repressor is C/EBP-γ. It is expressed in the pineal gland, has been shown to be degraded by the proteasome, and inhibits transcription from CCAAT elements.

another adrenergically induced gene, *mkp-1*; 5) the site of action of proteasomal inhibitors on adrenergic-stimulated aa-nat transcription is mediated at a post-cAMP step; 6) the accumulation of a protein repressor results in the inhibition of *aa-nat* transcription when proteasomal proteolysis is blocked 7) the protein repressor involved in the proteasomal inhibition effects on NE-stimulated *aa-nat* transcription is unlikely to be DREAM, Fra-2 or ICER. In addition, this study suggests that C/EBP-γ, a potential repressor of *aa-nat*, and its family members may be involved in the proteasomal regulation of *aa-nat* transcription in the rat pineal gland. Results from future research on members of the C/EBP family in pinealocytes may help to understand how the proteasome regulates the adrenergic induced transcription of *aa-nat*.

Not only will the results of this study help to understand the mechanisms that regulate the rhythmic transcription of *aa-nat*, but they will also contribute to the field of cAMP gene regulation. Like AA-NAT, a vast number of genes in a wide variety of cell types are regulated by cAMP dependent pathways. Data from this study may have implications for other researchers studying cAMP mediated gene expression.

6. Future Studies

1. Examine whether the mechanism of proteasome mediated gene repression described in this present study occurs in other cell types. Many processes in other cell types rely on cAMP signalling. In addition, maintaining a gene in a repressed state under basal conditions is a common regulatory mechanism for genes. Therefore, it will be important to examine whether proteasome mediated gene repression is a commonly used mechanism of gene regulation or whether it is only employed in certain tissue types, such as endocrine cells.

2. Determine what genes are regulated or not regulated by proteasome mediated gene repression. A thorough comparison of different gene promoters that are regulated and not regulated by the proteasome may uncover particular DNA elements that are responsible for mediating this type of repression. Since individual transcription factors only bind to their respective promoter sites, identifying a DNA element responsible for proteasome mediated gene repression will be important in determining its mechanism.

3. Determine if inhibition of the proteasome using MG132 or c-lact results in the accumulation of C/EBP-γ in rat pinealocytes. If blocking proteasomal proteolysis results in an increase in C/EBP-γ levels under non-stimulated conditions, this may suggest that C/EBP-γ is regulated by the proteasome in the pineal and that it could be a repressor of *aa-nat*.

4. It will be important to determine if C/EBP-γ binds to the *aa-nat* promoter using a chromatin immunoprecipitation assay (ChIP). Binding of C/EBP-γ to the *aa-nat* promoter would provide strong evidence that it regulates *aa-nat* transcription.

5. It would also be very interesting to knockout C/EBP-γ function using RNA interference (RNAi). This would involve designing a short-hairpin RNA (shRNA) that is complementary to C/EBP-γ and then inserting it into an adenovirus vector. Production of a functional adenovirus that expresses the shRNA would result in the degradation of C/EBP-g mRNA and effectively silence the gene. The adenovirus could be used to knockout C/EBP-γ in rat pinealocytes and then it would be easy to assay for *aa-nat* expression using RT-PCR, Western blot and enzymatic activity.

6. Another gene of interest is *icer* since it has been shown to repress *aa-nat* transcription and it is also regulated by the proteasome. It would be possible to silence *icer* expression using a similar adenovirus vector to the one used for C/EBP-γ. The only difference would be that the ICER adenovirus would contain an shRNA that is complementary to *icer* mRNA. Knocking out *icer* expression in rat pinealocytes would help determine whether the repressor plays a role in repressing *aa-nat* under basal conditions or if it is mostly responsible for suppressing *aa-nat* transcription at the end of the night.

7. Since ICER and C/EBP-γ are regulated by the proteasome, it would also be important to show that blocking proteasomal proteolysis would result in the accumulation of polyubiquitinated versions of both repressors in rat pinealocytes. This could be done by immunoprecipitation of the proteins with their respective antibodies, transferring them to a Western blot and then probing them with an antibody that recognizes polyubiquitinated proteins.

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